PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/53, 33/536, C12N 15/63, C12Q 1/68, C07H 21/04 — A1

(11) International Publication Number: **WO 99/38008**

(43) International Publication Date: 29 July 1999 (29.07.99)

(21) International Application Number: PCT/US99/01331

(22) International Filing Date: 22 January 1999 (22.01.99)

(30) Priority Data:
60/072,253 23 January 1998 (23.01.98) US

(71) Applicant: PROLIFARON, INC. [US/US]; Suite K, 11494 Sorrento Valley Road, San Diego, CA 92121 (US).

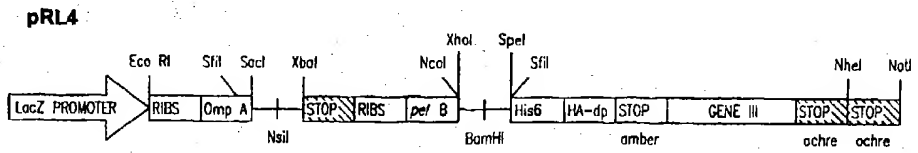
(72) Inventor: BOWDISH, Katherine, S.; 13754 Boquita Drive, Del Mar, CA 92014 (US).

(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon LLP, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071-2066 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHODS AND COMPOSITIONS FOR THE IDENTIFICATION OF GROWTH FACTOR MIMETICS, GROWTH FACTORS AND INHIBITORS



(57) **Abstract**

Method to identify agonist or inhibitory antibodies to receptors involved in cellular proliferation, differentiation, survival or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the receptors, so as to generate a plurality of cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of cells, comprising nucleic acid sequences encoding the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the antibodies are surface displayed, and screening the surface displayed antibodies using target cells to identify agonist or inhibitory antibodies to the receptors.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

DESCRIPTION

METHODS AND COMPOSITIONS FOR THE IDENTIFICATION OF GROWTH FACTOR MIMETICS, GROWTH FACTORS AND INHIBITORS

FIELD OF THE INVENTION

The present invention relates to the field of growth factors, inhibitors and their receptors.

BACKGROUND OF THE INVENTION

The following is a discussion of the relevant art, none of which is admitted to be prior art to the appended claims.

Conventional means, i.e., protein purification and expression screening have lead to the identification of a limited number of growth factors and cytokines.

In hematopoiesis, for example, cytokines are required for development of all mature, highly specialized blood cells. All blood cells are believed to derive from a common pool of pluripotent stem cells which are able to undergo self-renewal or give rise to progenitor cells. Cytokines control the complex process involving continuous coordinated differentiation and proliferation of stem and progenitor cells. Many cytokines are locally produced by bone marrow stromal cells. Others are produced in other areas of the body. Some cytokines have broad specificity, acting on pluripotent stem cells leading to their differentiation, self-renewal, and proliferation. Others act late in hematopoiesis on cells of particular lineages. Many cytokines also affect the activity of mature cells, playing a role in the immune response to extrinsic antigens. Many of the known cytokines and their major roles in hematopoiesis are described in Table 1 (Metcalf, D., and Nicola, N.A. 1995. In *The Hemopoietic colony-Stimulating Factors.* Cambridge University press, New York; Callard, R.E., and Gearing, A.J.H. 1994. In *The Cytokine Facts Book.* Academic Press Inc., San Diego, CA; Hamblin, A.S. 1993. In *Cytokines and Cytokine Receptors.* Oxford University Press Inc., New York).

TABLE 1

IL-3	Broad specificity, acts on pluripotent stem cells for differentiation, self renewal, and proliferation. Myeloid progenitors develop into early erythrocytes, neutrophils, eosinophils, basophils, macrophages, and megakaryocytes.
GM-CSF	Broad specificity, acts on pluripotent stem cells for differentiation, self renewal, and proliferation. Gives rise to neutrophils, macrophages, and eosinophils.
G-CSF	Acts late in hematopoiesis to promote development of primarily neutrophils and their precursors.
M-CSF	Acts late in hematopoiesis to promote macrophage development
Erythropoietin	Produced by the kidney. Responsible for terminal erythrocyte development and regulation of red cell development. Stimulates erythrocytes and megakaryocytes to develop in presence of IL-3 or GM-CSF.
IL-1	Primes stem cells to become responsive to CSFs. Induces other cells to produce GM-CSF
IL-2	Promotes T cell division and activation of NK and B cells.
IL-4	Promotes mast cell production.
IL-5	Promotes eosinophil differentiation.
IL-6	Induces B cell differentiation.
IL-7	Produced by bone marrow and thymic stromal cells. Is important in the proliferation and differentiation of pro-B cells and early thymocytes.
IL-8	Activated neutrophils.
IL-9	Stimulates T cell proliferation. Differentiation factor for erythrocytes.
IL-10	Inhibits cytokine production.
IL-11	Differentiation factor for megakaryocytes.

SCF (Kit-ligand)	Promotes colony formation from early hematopoietic progenitor cells by synergising with other growth factors. Promotes mast cell proliferation. Promotes survival of hematopoietic stem cells, and other cell types. Plays roles in cell adhesion and migration.
TNF- α, β	Enhances B and T cell proliferation, activates granulocytes and macrophages, induces cytokine secretion.
gamma-interferon	Enhances B cell proliferation and differentiation, activates macrophages, increases secretion of other cytokines.
LIF	Supports survival of hematopoietic stem cells.
TPO	Stimulators platelet production.
flk2/flt3 ligand	Stimulates proliferation of early hematopoietic cell precursors.

Besides natural growth factors and cytokines, agonist antibodies have been discovered. Agonist antibodies are antibodies that mimic the natural ligand of a receptor. Agonist antibodies have been identified in hematopoiesis and related growth and differentiation pathways, using conventional monoclonal antibody technology. Agonist antibodies have been identified against Hepatoma transmembrane kinase (Htk), a tyrosine kinase in $CD34^+$ human bone marrow cells and a human hepatocellular carcinoma cell line (Bennett, B.D., Wang, Z., Kuang, W.J., Wang, A., Groopman, J.E., Goeddel, D.V., Scadden, D.T., *J. Biol. Chem.* 269:14211-14218, 1994). Agonist antibodies have also been raised to flt3/flk2 (Bennett et al. U.S. Patent No. 5,635,388).

An antibody that mimics erythropoietin has been identified through screening of monoclonal antibodies generated against the erythropoietin receptor. This antibody promotes receptor response via dimerization (Schneider et al., *Blood* 89:473, 1997). Other antibodies that mimic ligands have also been identified (Kahan et al. *Proc. Natl. Acad. Sci., USA* 75:4209, 1978).

In some disease states antibodies that bind to receptors can mimic the effect of a natural ligand. In hyperthyroid disease (Graves disease) there is the abnormal production of antibodies that bind to TSH receptors and activate these receptors (Kosugi, S., Ban, T., Kohn, L.D., *Mol. Endocrinol.* 7:114-130, 1993; Lundgate, M.E,. Vassart, G., *Baillieres Clin. Endocrinol.* Metab. 9:95-113, 1995).

In contrast to antibodies that promote proliferation or differentiation, inhibitory antibodies have been identified that bind to cell surface receptors and efficiently interfere with ligand binding. These antibodies act by competing with the native molecule for the same binding site, or by blocking the native binding site by binding to a site in close proximity. There are many examples in the literature of these types of inhibitory antibodies, including antibodies against the c-fms receptor (Sudo, T., Nishikawa, S., Ogawa, M., Kataoka, H., Ohno, N., Izawa, A., Hayashi, S., Nishikawa, S., *Oncogene* 11: 2469-2476, 1995), and against the c-kit receptor (Kodama, H., Nose, M., Niida, S., Nishikawa, S., Nishikawa, S., *Exp. Hematol.* 22: 979-984, 1994).

Alternatively, some inhibitory antibodies may act by mimicking a native inhibitory molecule. Cytokines with inhibitory effects on hematopoiesis have been identified (Quesenberry, P.J. 1995. Hemopoietic stem cells, progenitor cells, and cytokines. In *Williams Hematology*, Fifth Edition. Eds. Beutler, E., Lichtman, M.A., Coller, B.S., Kipps, T.J. McGraw-Hill United States). These inhibitory molecules act by suppressing cell division during S phase, modulating surface cytokine receptor expression, or by suppressing release of cytokines from cells. Transforming growth factor-B (TGF-B) inhibits early stem cells while stimulating more mature cells. Other cytokines with inhibitory effects include H-subunit ferritin, Prostaglandin E1 and E2, Inhibin, and Lactoferrin (Quesenberry 1995). Inhibitors of hematopoiesis in the chemokine family include macrophage-inflammatory protein-1a, macrophage-inflammatory protein-2a, platelet factor-4, interleukin-8, interferon inducible protein-10, as well as a few small peptides (Quesenberry, 1995). Action of the inhibitor may require dimerization of a receptor, or a conformational change of a receptor triggered by binding. An inhibitory antibody may mimic an inhibitory ligand similarly by promoting dimerization or change in receptor conformation.

SUMMARY OF THE INVENTION

The present invention utilizes an immune system approach for the identification of new growth factor mimetics, growth factor receptors, native growth factors, growth factor receptor antagonists, and inhibitors involved in differentiation pathways, developmental pathways, cell survival and functional activation or inhibition of cells. Such pathways include, but are not limited to, hematopoiesis, nervous system development and regeneration

and organ/tissue development and regeneration. In a preferred embodiment the invention concerns the identification of growth factors, mimetics and inhibitors that affect growth and differentiation of hematopoietic cells including pluripotent stem cells, multipotent progenitor cells and unipotent cells at various stages within each hematopoietic lineage including granulocytes, monocytes, macrophages, eosinophils, megakaryocytes, mast cells, erythroid cells, T-lymphocytes, B-lymphocytes and dendritic cells.

The broad range screening and identification methods of the current invention allow for the selection and screening through a large repertoire of binding molecules to cell surface molecules to find those that have proliferative, differentiative, developmental, cell survival, functional activation or functional inhibitory effects. In preferred aspects the method uses combinatorial libraries and phagemids to identify first generation mimetic molecules (agonist antibodies) or inhibitor molecules (inhibitory antibodies) (e.g. antibodies that mimic natural inhibitors or antagonist antibodies) that behave as agonists or inhibitors in proliferation, differentiation, survival or activation of various cell lineages. First generation agonists and inhibitors can be used directly as therapeutic agents (e.g., for agonists: amplification of clinically relevant cell types; for *ex vivo* proliferation and differentiation for gene therapy purposes; e.g., for inhibitors: inhibition of the proliferation of normal cells only so that cancerous cells will continue to divide and be more susceptible to chemotherapeutic agents or inhibition of the proliferation or differentiation of a population of cells that is involved in a disease or disorders, such as cells involved in allergic reactions), as reagents in diagnostics, and in basic and clinical research (e.g., antibodies for cell sorting of cells such as hematopoietic cells and for the identification of cells, such as hematopoietic cells). First generation agonists and inhibitors can also be used to clone the receptors, and ultimately, the native factors they mimic. Native factors that result from these studies could be used clinically in many disease states. For example, beneficiaries of new hematopoietic growth factors and mimetics include, but are not limited to, all patients, including HIV infected patients that suffer from disease or treatment related immunosuppression, including chemotherapy, bone marrow transplants, and myeloproliferative disorders, and will augment the armamentarium of this class of therapeutic agents.

One aspect of the invention involves the immunization of an animal with human or murine stem/progenitor cells so as to generate immune repertoire libraries of antibodies raised to epitopes displayed on the surface of these cells. As mixtures of primary cells are utilized, immunogens will be expressing thousands of potential epitopes which represent portions of cell surface receptors involved in a variety of different stages of development, differentiation, survival, activation and inactivation for a variety of cell types. The epitopes of interest are those cell surface receptors that link to a signal transduction cascade that lead to enhancement or inhibition of proliferation, differentiation, development, survival or activation. For example, to raise antibodies that effect cells of hematopoietic origin, bone marrow cells would be one choice of cells to be injected.

The present methods of discovery of new growth factors, inhibitors and related molecules is applicable to other differentiation/developmental pathways in addition to hematopoiesis. For example, an animal (rabbit, chicken, or other animal) can be immunized with murine or human embryonal carcinoma cells, such as murine P19 cells (McBurney, M.W., Rogers, B.J., *Developmental Biology* 89:503-508, 1982) or human NTera-2c1.D1 cells (Andrews, P.W., Damjanov, I., Simon, D., Banting, G.S., Carlin, C., Dracopoli, N.C., Fogh, J., *Laboratory Investigation* 50:147-162, 1984). These cells are pluripotential, but differentiate poorly under normal culture conditions. They can be induced to differentiate into neuronal and glial cells (generally in presence of inducing agents such as retinoic acid), or cardiac muscle and skeletal muscle (for example, in the presence of DMSO). Immunization with these cells will allow for the identification of agonist antibodies, inhibitory antibodies and growth factors that are involved in differentiation into these and other pathways.

Alternatively, an animal could be immunized with human or mouse pluripotent teratocarcinoma cells. In this case, agonist or inhibitory antibodies could be identified that would promote or inhibit development of endodermal derived tissues and organs, such as lung, liver, pancreas, stomach, esophagus, pharynx, intestines, or salivary glands.

Immunization is also possible with murine pluripotent embryonic stem cells. Embryonic stem cells (ES cells) are derived from early mammalian cells that are totipotent and capable of *in vitro* proliferation. They can differentiate to all three embryonic germ layers and their derivatives. (Evans, M.; Kaufman, M., *Nature* 292: 154, 1981; Martin, G., *Proc.*

Natl. Acad. Sci. U.S.A.,78: 7634, 1981). In addition, human ES cell lines could be used as immunogens (Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swwiergiel, J.J., Marshal, V.S., Jones, J.M., *Science* 282: 1145-1147, 1998; Bongso, A., C.Y. Fong, S.C. Ng, and S. Ratnam, *Hum. Reprod.* 9: 2110, 1994). In this case, agonist or inhibitory antibodies could be identified that promote or inhibit differentiation into a variety of embryonic structures.

Following immunization, combinatorial antibody fragment libraries are constructed from an immunized animal. The present invention also encompasses these combinatorial libraries directed to surface components on the various cells used for immunization. These libraries will yield a higher frequency of specific antibodies to antigens of interest as compared with naive libraries or synthetic libraries. Libraries generated from an animal that has undergone 1-3 booster immunizations will also result in antibody fragments with higher affinities, due to affinity maturation.

Alternatively, in a less preferred embodiment existing antibody libraries, such as synthetic antibody phage display libraries, may be screened. However, as these are not targeted libraries, agonist or inhibitory antibodies of interest may not be present. A number of synthetic antibody phage display libraries contain a broad assortment of binding specificities. Libraries have been created using random oligonucleotide synthesis (Barbas, C.F. III, Bain, J.D., Hoekstra, D.M., Lerner, R.A., *Proc. Natl. Acad. Sci U S A* 89: 4457-4461, 1992; Soderlind, E., Vergeles, M., Borrebaeck, C.A., *Gene* 160: 269-272, 1995). In addition, large phage display libraries of human single chain Fv (scFv) antibody fragments were constructed by combining germline VH genes with synthetic heavy chain CDR3 regions and various light chain sequences (de Kruif, J., Boel, E., Logtenberg, T., *J. Mol. Biol.* 248: 97-105, 1995; Akamatsu, Y., Cole, M.S., Tso, J.Y., Tsurushita, N., *J Immunol.* 151: 4651-4659, 1993). Other synthetic libraries have also been reported (Fuchs, P., Dubel, S., Breitling, F., Braunagel, M., Klewinghaus, I., Little, M., *Cell Biophys.* 21: 81-91, 1992; Hoogenboom, H.R., and Winter, G., *J Mol. Biol.* 227: 381-388, 1992).

In the present invention, expression libraries from an immunized animal are preferably constructed as either Fab fragment libraries or single chain variable region libraries (scFv). Fab fragment libraries, that maintain the native antigen recognition site, are useful to ensure

that affinity is maintained. Single chain libraries are useful because the entire binding domain will be contained on one polypeptide.

Antibody or antibody fragments can be presented to target cells for screening purposes in a variety of ways. First, they can be surface displayed on bacteriophage, phagemids, prokaryotic cells such as *E. coli*, eukaryotic cells such as mammalian cells or yeast or displayed on ribosomes. The surface displayed antibodies are screened by contacting the entity that carries the surface displayed antibody (bacteriophage, phagemid, bacterial cell or mammalian cell) with the target cells so that binding to target cells occurs, removing unbound entities, amplifying any entities that remain bound for further testing in bioassays. Alternatively, co-cultivation can be used for screening purposes. The antibody producing cells, bacterial cells producing bacteriophage or phagemids, bacterial cells producing antibodies, eukaryotic cells producing antibodies) are grown in the presence of target cells (co-cultivation). Antibodies are presented on the surface of cells, bacteriophages or phagemids or are secreted into the medium. A third, means of presentation to target cells is by utilizing secreted antibodies.

Antibody display has been done on the surface of bacteriophage (Huse, W.D., Sastry, L., Iverson, S.A., Kang, A.S., Alting-Mees, M., Burton, D.R., Benkovic, S.J., and Lerner, R.A., *Science* 246:1275-1281,1989; McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J., *Nature* 348:552-554, 1990; Chang, C.N., Landolfi, N.F., and Queen, C., *J., Immunol.* 147:3610-3614, 1991); phagemids (Barbas III, C.F., Kang, C.F., Lerner, R.A., and Benkovic, S.J., *Proc. Natl. Acad. Sci., USA*, 88: 7978-7982, 1991) and on the surface of prokaryotic cells, such as *E. coli* (Fuchs, P., Breidling, F., Belscheaus, T., Little, M., *Biotechnology* 9: 1369-1372, 1991; Francisco, J.A. et al., *Proc. Natl. Acad. Sci., USA*, 90: 10444-10448, 1993; Chen, G., Cloud, J., Georgiou, G., Iverson, B.L., *Biotechnol. Prog.* 12: 572-574, 1996). Antibodies have been expressed intracellularly and secreted in eukaryotic cells. Antibodies have also been expressed on the surface of eukaryotic cells (yeast). Mammalian CHO cells and COS cells have been well utilized for antibody secretion (Trill, J.J., Shatzman, A.R., Ganguly, S., *Curr. Opin. Biotechnol.* 6: 553-560, 1995; Fouser, L.A., Swanberg, S.L., Lin, B.Y., Benedict, M., Kelleher, K., Cumming, D.A., Riedel, G.E., *Biotechnology (N Y)* 10: 1121-1127, 1992). The GPI anchor is well utilized for anchoring a variety of proteins to the

cell surface. In eukaryotic cells, antibody fragments could be displayed on the surface of the plasma membrane using a GPI anchor. Fusion proteins linked to a GPI anchor have been used extensively for the expression of heterologous proteins on the cell surface (Scallon, B.J., Kado-Fong, H., Nettleton, M.Y., Kochan, J.P., *Biotechnology* 10: 550-556, 1992). In yeast, antibody expression has been done intracellularly (Carlson, J.R. and Weissman, I.L., *Mol. Cell. Biol.*, 8:2647-2650, 1988; Bowdish, K. S., Tang, Y. Hicks, J.B., and Hilvert, D., *J. Biol. Chem.* 266: 11901-11908, 1991) and on the surface (Boder, E.T. and Wittrup, K.D., *Nat. Biotechnol.*, 15: 553-557, 1997)). A variety of stable vectors and efficient promoters and secretion signals are available in yeast for engineering the secretion of any protein of interest. These have been reviewed in Moir, D.T., Davidow, L.S., *Methods Enzymol.* 194: 491-507, 1991. Cell surface expression of heterologous proteins on the surface of yeast was reviewed recently (Georgiou, G., Stathopoulos, C., Daugherty, P.S., Nayak, A.R., Iverson, B.L., Curtiss, R. III., *Nat. Biotechnol.* 15: 29-34, 1997). Cloning fragments downstream from the leader sequence Mating Factor alpha has been used successfully for secretion of heterologous proteins (Swart, A.C., Swart, P., Roux, S.P., van der Merwe, K.J., Pretorius, I.S., Steyn A.J., *Endocr. Res.* 21: 289-295, 1995). In addition, numerous heterologous proteins have been produced at greater than gram per liter levels in the methylotropic yeast *Pichia pastoris* using the methanol oxidase promoter (Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J.T., Smith, P.L., Wierschke, J.D., Subramaniam, A., Birkenberger, L.A., *Gene* 190: 55-62, 1997). Those of ordinary skill in the art based on these and other known techniques can readily achieve surface display and secretion of antibody molecules and fragments.

Those who practice the art appreciate that other means for presenting antibodies or antibody fragments to target cells that are not surface display or secreted are possible and are suitable for use in the present invention. These methods include, but are not limited to, prokaryotic ribosome display (Hanes, J. and Pluckthun, A., *Proc. Natl. Acad. Sci. U.S.A.*, 94: 4937-4942, 1997) and eukaryotic ribosome display (Translocus Therapeutics, Cambridge, United Kingdom).

Preferably, the libraries are constructed in phagemid vectors which allows for rapid screening. Phagemids require superinfection with helper phage. Superinfection will provide

the remaining phage components needed for packaging plasmids into phagemid particles. Each phagemid will contain approximately one or more antibody binding sites, displayed on the surface as a coat protein fusion, per phagemid particle. The remaining coat proteins will be contributed by helper phage and will therefore be wildtype and allow for efficient reinfection of phagemids into *E. coli* for amplification. The functional domain of the gene III coat protein is preferred as the fusion partner for display of the antibody fragment. However, it is also possible to utilize any full length or functional domain coat protein of the phage, such as gene VIII, that allows for phage surface display (Kang, A.S., Barbas III, C.F., Janda, K.D., Benkovic, S.J., and Lerner, R.A. *Proc. Natl. Acad. Sci. USA*, 88:4363-4366, 1991).

The initial screening of the surface displayed antibody molecules or fragments is preferably by panning (DeKruif, J., Terstappen, L., Boel, E., Logtenberg, T., *Proc. Natl. Acad. Sci. USA* 92: 3938-3942, 1995). Panning of monomeric antigen binding sites has the advantage of sorting clones based on affinity as well as specificity, and will therefore skew the population towards isolation of high affinity binders. Typically there are several rounds of selection and amplification so as to enrich for binding molecules. Panning is conducted utilizing target cells expressing the cell surface receptor toward which the antibody is directed or a closely related receptor. Alternatively, cells can be separated by fluorescence activated cell sorter (FACS) sorting or magnetic sorting. Phage that adhere to a specific population of cells can be grown out by eluting and infection of bacterial cells.

Another alternative for initial screening detects binding of antibody molecules or fragments surface displayed on phage to target cells expressing the cell surface receptor toward which the antibody is directed or a closely related receptor and which results in the bound phage become internalized by endocytosis of the receptor. These phage can be identified, for example, by eluting surface bound phage and lysing cells followed by electroporation to recover internalized phage DNA.

Another possible method of identification of specifically bound antibodies involves the use of radiolabelled cell lysates or membrane preparations which are electrophoresed in one or two dimensional gels and transferred to an immobilized solid support membrane. Phage are hybridized and eluted from specific spots of interest. Spots can be chosen based

on comparing the pattern of membrane proteins exhibited by targets cells which have the cell surface molecules of interest and unrelated cells which should not.

Surface displayed antibodies that specifically bind to target cells (those that remain on the surface of the target cell or those that are internalized) are then screened against various target cells and cell lines for the ability to promote or inhibit proliferation, differentiation, cell survival, or activation utilizing various bioassays or receptor assays. Dimerization is often a prerequisite for activation of many receptors including hematopoietic receptors (The Hematopoietic Colony-Stimulating Factors, D.M. Metcalf, N.A. Nicola (1995) Cambridge University Press, New York). Thus, subsequent to panning, antibody fragments identified as binding cell surface molecules on target cells are tested in bioassays or receptor assays as dimers. Inhibitory antibodies that act as antagonists may not need to be dimers, especially if they merely block the receptor from binding of an agonist, however if the inhibitory antibody mimics a native inhibitory molecule it may be required to be a dimer to be functional.

The present invention offers several advantages over other methods for identification of growth related molecules. Prior approaches to the isolation of growth factors utilized purification of active factors from conditioned medium by separative biochemistry. These methods required the factors to be present in significant quantities. Other methods use direct expression screening of cDNA pools, using cell lines as bioassays. In this case, the level of transcription is a factor in successful identification of the factor, as is proper folding of the polypeptide chain in the expression screen. Also, it is increasingly becoming obvious that many of the regulators were designed to function most efficiently when acting in combination, these approaches are limited in that they can only identify factors that act singly to affect proliferation and/or differentiation.

In distinction, the present invention selects directly for binding molecules and then screens through the entire repertoire of binding molecules to find those with proliferative or differentiative effects. A large immune repertoire as a combinatorial library, allows for random association of heavy and light chain binding regions, increasing candidate agonists and inhibitors even further. Also, the claimed methods are not subject to limitations of traditional monoclonal antibody technology (limited by the number of B cell fusions to myeloma cells per immunized animal) and will enable screening through a large immune

repertoire. In addition, the screening methods are not limited to the type of regulator or to its origin or site of production, as it is known that both local control through cell-cell contact, and humoral regulatory molecules are involved in proliferation, development, differentiation, cell survival, functional activation and functional inhibition. Furthermore, the present screening methods do not presuppose any biochemical function, extensive sequence similarity with known genes, or site of origin. The claimed identification and screening methods offer several practical advantages over conventional screening methods including: rapidity, cost-effectiveness, and simplicity. The current methods also offer simultaneous multi-sample analysis, which renders more Ab fragments available for screening.

The present invention also encompasses methods for screening antibodies (agonist or inhibitory) by co-culturing cells that express the antibodies in the presence of cells (target cell) that express receptors that the antibodies bind.

Once agonist or inhibitory antibodies are identified they can be synthesized using standard recombinant techniques known to those who practice the art. These antibodies and the methods of making them, are also encompassed by the claimed invention. The present invention further encompasses methods for the use of such antibodies to identify the receptors to which the antibodies bind and to identify the native growth factors or inhibitory factors that the antibodies may mimic.

Furthermore, the present invention encompasses use of agonist antibodies to treat a patient with a deficiency in a particular cell type by stimulating the proliferation or differentiation of the cell type or its precursors and the use of inhibitory antibodies to inhibit a particular cell type, and the use of antibodies to identify particular cell types.

Also, encompassed by the present invention is use of agonist and inhibitory antibodies to identify and isolate specific populations of cells.

In a first aspect, the invention features a method to identify agonist or inhibitory antibodies to receptors involved in cellular proliferation, differentiation, survival or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells comprising nucleic acid sequences encoding the antibodies, cloning the nucleic

acid sequences from the library into surface display vectors so that the antibodies are surface displayed, and screening the surface displayed antibodies using target cells to identify agonist or inhibitory antibodies to the receptors.

By "receptor involved in cellular proliferation, differentiation, survival or activation" is meant a receptor linked to a signal transduction cascade that leads to enhancement or inhibition of proliferation, differentiation, development, survival or activation of the cell that displays the receptor. Proliferation is active division and progression through the various stages of the cell cycle as detected by changes in the rate of protein synthesis, chromosome replication, cell size or cell number. Differentiation involves changes in cell morphology, behavior or function that lead to the production of different types of cells with specialized functions, as a result of exposure to extrinsic factors, or changes in gene expression by which cells mature and become less pluripotent. Activation is the process by which cells leave G_0 and enter G_1, but do not synthesize DNA or divide until a second signal is received. Activation involves the expression of a specific set of activation genes and activation antigens. For example, activation antigens for B cells include CD23, surface IgM, and CD40. Survival is meant that cells do not undergo apoptosis or programmed cell death or necrosis. Although the present invention encompasses antibodies directed to yet undiscovered receptors, the identity of the receptors is not necessary as one of ordinary skill in the art would be able to detect binding of the antibodies to the receptors by the resultant behavior exhibited by the cell, i.e., proliferation, differentiation, activation, or survival.

In preferred embodiments the stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and

NTera-2 pluripotent embryonal carcinoma cells, the antibodies are scFv or Fab fragments, the surface display vectors are phagemids, the library is a combinatorial library.

In another embodiment, the invention features a method to identify agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells comprising nucleic acid sequences encoding the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the antibodies are surface displayed, and screening the surface displayed antibodies using target cells to identify agonist antibodies to growth factor receptors.

By "agonist antibody" is meant entire antibodies or fragments thereof, including Fab fragments and scFv fragments, that bind to a cell surface receptor that is linked to a signal transduction cascade that leads to the proliferation, differentiation, activation, survival or preservation of the viability of the cell that displays the cell surface receptor.

By "growth factor receptor" is meant a molecule that binds to a growth factor or another ligand and participates in a signal transduction cascade that leads to the proliferation, differentiation, survival or activation of a cell which has the growth factor receptor presented on its plasma membrane (i.e., cell surface).

By "immunizing an animal" is meant injecting one or more animals by any route of administration, e.g., intraperitoneally, intravenously, subcutaneously, or intramuscularly with cells, e.g., stem/progenitor cells, so that an immune response is generated by the animal to molecules on the surface of the cell. Primary immunizing can be in the presence of adjuvant. Primary immunization is often followed by boosts with the same antigen to produce a greater response and increased affinity of antibodies produced. Typically, 1 to 3 booster immunizations are given at intervals of 2-8 weeks, usually at 3-4 weekly intervals. Typically, more than a single animal is immunized.

By "stem/progenitor cells" is meant to include cells that are pluripotent and cells that are multipotent and also end stage or terminally differentiated cells derived from pluripotent or multipotent cells that have cell surface receptors involved in cellular proliferation, differentiation, activation or survival. Pluripotent cells (stem cells) are able to differentiate

into all cell lineages. For example, human embryonic stem (ES) cells are able to differentiate to all three embryonic germ layers and their derivatives. The least mature hematopoietic stem cells are able to form mature hematopoietic cells of all lineages and repopulate the hematopoietic tissues of an animal. Multipotent cells (progenitor cells) are restricted to differentiating to a limited number of lineages (typically two or three). Hematopoietic stem/progenitor cells are present in bone marrow and in circulating blood. Stem/progenitor cells include, but are not limited to, unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells. Cells for immunization can be obtained from any of these sources.

By "surface molecules" is meant plasma membrane (cell membrane) protein molecules where at least a portion of the molecule is on the extracellular face of the plasma membrane (cell membrane).

By "plurality of immune cells expressing one or more antibodies" is meant a collection of antibody producing cells from blood or lymphoid organs, such as bone marrow, spleen and lymph nodes from an immunized animal.

By "library" is meant a collection of nucleic acid molecules representing the immune repertoire of an organism, which in the present invention would include molecules encoding for antibodies and fragments directed toward the growth factor receptors of the relevant stem/progenitor cell. Libraries are typically presented as nucleic acid molecules cloned in a vector, e.g. surface display vector.

By "surface display vector" is meant a nucleic acid vector that carries at least the functional domain of a surface display protein, i.e., the leader sequence that directs export

from the cells, and a cloning site to insert antibody sequences, which results in an antibody fusion gene. The surface display vector when in a host cell allows for the production of a fusion protein consisting of a surface display protein or functional domain thereof and an antibody or fragment thereof so that the antibody or fragment is surface displayed. That is the antibody or fragment is presented on the surface as a functional binding polypeptide, linked to the domain of the surface display protein. Surface display can be on the surface of a bacteriophage, phagemid, bacterial cell, or mammalian cells. Examples of surface display vectors include, but are not limited to, pCOMB3, SurfZAP, pCANTAB5E and pEXmide 3. Those who practice the art are familiar with these and other surface display vectors and can readily construct other similar vectors. Antibodies that are surface displayed are able to bind antigen presented from the extracellular side of the plasma (cell) membrane in which they are located.

By "screening" is meant using methods for identifying antibodies that are agonist antibodies or are inhibitory antibodies. For agonist antibodies, screening involves identifying antibodies that bind to receptors on target cells, so as to trigger or activate a receptor that participates in a signal transduction cascade that leads to the proliferation, differentiation, survival or activation of that cell. Screening may involve only the determination of the ability of the antibody to bind to the receptor on a target cell (including both antibodies that remain on the cell surface after binding and those that are internalized after binding) and/or assays that determine cellular responses associated with binding of the antibody to the receptor; these include bioassays that detect differentiation, proliferation, cell survival or activation (such as changes in transcription of downstream effector genes) and biochemical assays that detect chemical processes such as phosphorylation. For inhibitory antibodies screening involves identifying antibodies that bind to receptors on cells, such that the binding of the antibody to the receptor results in the inhibition of growth, proliferation, differentiation, activation or survival of that cell. Screening may involve only the determination of the ability of the antibody to bind to the receptor on a cell (including both antibodies that remain on the cell surface after binding and those that are internalized after binding) and/or assays that determine cellular responses associated with binding of the antibody to the receptor; these include bioassays that detect differentiation, proliferation, cell survival or activation (such as changes

in transcription of downstream effector genes) and biochemical assays that detect chemical processes such as phosphorylation.

By "target cells" is meant cells that display receptors to which agonist or inhibitory antibodies bind. The growth factor receptor on the target cell is the same as or highly related to the receptor present on the stem/progenitor cell. For hematopoietic cells, these may include, but are not limited to, primary hematopoietic cells, several factor dependent murine cell lines and human leukemia cell lines. Target cells could be the same primary cell type, a population of cell types, or cell line used as an immunogen or another cell type or cell line. Target cells are chosen based on the immunogen used. For example, immunizing broadly with human primary bone marrow aspirates treated with red blood cell lysing reagents and centrifugation to remove red blood cells and platelets should result in antibodies with specificities against a wide variety of cell surface receptors. Target cells in this case could include the same immunogen where a large variety of antibodies would be isolated, or target cells could include a sorted population of cells, such as $CD34^+$ cells to identify antibodies against a specific cell lineage. Another target option with the broad immunogen would be to utilize the murine hematopoietic cell line FDCP-mix. In this case, a more uniform population of cells, capable of *in vitro* growth has its advantages, however, agonist antibodies identified using a murine target would have to be further characterized for its ability to promote effects on human cells. Alternatively, with an immunogen composed of a sorted population of cells, such as $CD34^+$ cells, one would likely use the $CD34^+$ cells as the target, or the FDCP-mix cell line. Thus, the appropriate choice of immunogen and target cell are key to identifying relevant antibodies. Those of ordinary skill in the art would readily be able to determine the appropriate target cell based on the nature of the antibody sought, i.e., an antibody directed to a receptor that only participates in the growth of precursor cells would require the target cells to be early lineage cells.

In a preferred embodiment, the invention features a method to identify agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of cells, comprising nucleic acid sequences

encoding the antibodies, cloning the nucleic acid sequences from the library into viral display vectors so that the antibodies are displayed on the surface of virus, and screening the antibodies displayed on the surface of the virus using target cells to identify agonist antibodies to the growth factor receptors.

By "viral display vectors" is meant vectors that allow for display of an antibody or fragment thereof on the surface of a virus. Preferably the viral vectors are bacteriophage (e.g., f1, M13, fd, λ, T3, T4, T7) or phagemids vectors. Bacteriophage are viruses able to infect bacterial cells, replicate and package their genome and upon release from a cell are able to continue this cycle by infecting other cells. Phagemids are virus that are missing essential genes required for the above described life cycle, which are provided by separate helper phage. Those of ordinary skill in the art are familiar with viruses, bacteriophage and phagemid vectors which are suitable for use in the claimed invention. Examples of viral display vectors include pRL4 (Prolifaron, LLC, San Diego, CA), pCOMB3 (Burton, D.R. and Barbas, C.F. III. *Advances in Immunology* 57:191-280, 1994), SurfZAP Vector (Stratagene, La Jolla, CA), pCANTAB5E (Pharmacia, Piscataway, NJ), pEXmide 3 (Soderlind, E., Lagerkvist, A.C., Duenas, M., Malmborg, A.C., Ayala, M., Danielsson, L., Borrebaeck, C.A. *Biotechnology* 11: 503-507,1993).

In further preferred embodiments the viral vectors are selected from the group consisting of bacteriophage and phagemid vectors.

In another preferred embodiment, the invention features a method to identify agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, and screening using target cells, the scFv fragments displayed on the surface of the phagemid to identify those that are agonist antibodies for the growth factor receptors.

By "scFv fragment" is meant single chain antibody fragment whereby the complete antigen binding domain is contained within a single polypeptide.

In another preferred embodiment, the invention features, a method to identify agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the scFv fragments are surface displayed, and screening using target cells the surfaced displayed scFv fragments to identify those that are agonist antibodies for the growth factor receptors.

In a further preferred embodiment the surface display vector is a bacteriophage vector which allows for expression of the antibody on the surface of bacteriophage.

In another preferred embodiment, the invention features a method to identify agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, and screening in a functional assay the scFv fragments that bind the cell surface molecules to identify those that are agonist antibodies for the growth factor receptors.

By "panning" is meant exposing a surface display library (bacteriophage, phagemid or cell) to antigens of interest, i.e., cell surface molecules which include receptors to which the antibodies or antibody fragments are directed, which are displayed on target cells to enrich for antibodies that specifically bind to target cells. For example, the target cells can be immobilized, e.g., on a plastic surface, or fixed, or captured by centrifugation. After specific antibodies or fragments displayed on bacteriophage, phagemid or whole cells bind to target cells the remainder of the surface display library are removed by washing. Binding and washing are followed by elution (e.g., by low pH) of bacteriophage, phagemid, cells which

display specific binding antibodies. The panning process is typically repeated for several rounds. If bacteriophage or phagemid are utilized then, after each round eluted phagemid or bacteriophage may be amplified in host cells. Alternatively, fluorescence activated cell sorting (FACS), or magnetic cell sorting can be used for high throughput screening to identify relevant binding antibodies. Methods for FACS sorting of surface displayed molecules are described in: Georgiou, G., Stathopoulos, C. Daugherty, P.S., Nayak, A.R., Iverson, B.L., and Curtiss R. III. *Nature Biotechnology* 15: 29-34, 1996. These methods can be easily adapted to screen surface displayed antibody fragments by those of ordinary skill in the art. Panning for scSv fragments can be carried out as dimers, due to one cloning strategy employed, but could also be carried out as monomers. Alternatively, bacteriophage or phagemid that recognize receptors can also be identified using an internalization approach in which the bacteriophage or phagemid that remain bound to the cell surface are removed and then cells are lysed to recover internalized bacteriophage or phagemids. Another alternative, is to isolate membrane proteins from target cells and bind antibodies to specific fractions of these membrane proteins.

By "binding to cell surface molecules "is meant binding of the antigen binding domain of an antibody fragment to the antigenic determinant on the receptor to which it was raised or a closely related antigenic determinant, such as a determinant on a receptor of the same or a related family or a related receptor in another species.

By "screening in a functional assay" is meant examination of antibodies (agonist or inhibitory), that have been surface expressed, either individually or in pools to determine their effect on target cells (i.e., binding of antibodies to growth factor receptors displayed on target cells that effects proliferation, differentiation, development, survival or activation). Only antibody fragments that have been determined to bind to target cells by panning, are subsequently screened in the functional assays. In functional assays for agonist antibody fragments it is scFv dimers that are screened. Screening of the scFv dimer molecules can occur as surface displayed molecules, soluble scFv molecules or as secreted molecules. In functional assays for inhibitory antibody fragments the antibody fragments are screened independently as monomers and as dimers and as either surface displayed molecules or soluble molecules. If the scFv fragment acts as an antagonist, binding to a growth factor

receptor and blocking its activation, it may do so as a monomer. However, if the scFv fragment acts as a mimetic of a native inhibitory molecule, it may need to be a dimer as in the case of agonist antibodies. Screening in functional assays involves either bioassays (such as examination of colony formation, measurement of DNA synthesis by either ^{3}H thymidine incorporation or BrdU incorporation, assaying for changes in gene transcription, or measurement of cellular enzymes which can be used to screen for effects on proliferation) or biochemical assays (such as receptor phosphorylation). Those of ordinary skill in the art are familiar with bioassays and biochemical assays suitable for detecting cell proliferation, differentiation, survival and activation.

In another preferred embodiment, the invention features a method to identify agonist antibodies to growth factor receptors comprising the steps of an immunizing animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing the Fab fragments that bind the cell surface molecules, and screening the dimerized Fab fragments to identify those that are agonist antibodies for the growth factor receptors.

By "Fab fragment" is meant a fragment of an antibody where heavy chain variable region and the light chain variable regions are contained on separate polypeptides. The polypeptides are covalently bound to each other by at least one disulfide bridge. Constant regions present may be native or from different species, also known as hybrid or chimeric Fab.

Panning is carried out using target cells as previously described, although Fab fragments are generally monomers.

By "dimerizing" is meant causing the association of two antibody binding domains contained on antibody fragments so that binding can occur to more than one growth factor receptor. One way that dimerization can be achieved is by linking individual antibody fragments to dimerization domains such as jun (Kostelny, S.A., Cole, M.S., and Tso, J.Y. J.

Immunol. 148:1547-1553, 1992; deKruif, J. and Logtenberg, T. *J. Biol. Chem.* 271:7630-7634, 1996), which allow the two protein fragments to stably associate through the interaction of these domains.

"Screening in functional assays" is carried out using target cells as previously described for scFv fragments. The monomeric Fab molecules that bind to target cells are dimerized and screened as dimers. These can be surface displayed, but are preferably screened as soluble molecules.

In another preferred embodiment, the invention features a method to identify agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the Fab fragments are surface displayed, panning the surface displayed Fab fragments for binding to cell surface molecules on target cells, dimerizing the Fab fragments that bind the cell surface molecules, and screening in a functional assay the dimerized Fab fragments to identify those that are agonist antibodies for the growth factor receptors.

In a further preferred embodiment the surface display vector is a bacteriophage vector which allows for expression of the antibody on the surface of bacteriophage.

In other preferred embodiments of the methods to identify agonist antibodies, the animal is a rabbit or a chicken; the stem/progenitor cells are selected from the group consisting unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem

cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

The choice of preferred animals is twofold: Rabbits are routinely used successfully for production of antibodies and are generally the first choice, provided that species differences with regard to the immunogen will permit generation of an immune response. However, chickens provide a more evolutionarily distinct environment in which to elicit antibodies. This is an important consideration because rabbits and mice do not elicit an immune response to some human antigens. However, those skilled in the art are familiar with other animals which are suitable for use.

Cells useful in the present invention include primary cells isolated from an animal, cells cultured *in vitro* from primary cells and established cell lines that can be grown continually in culture.

By "unsorted human bone marrow cells" is meant samples of human bone marrow aspirates that have undergone minimal manipulation, for example aspirates treated to lyse red blood cells and to remove platelets by centrifugation, such that all other lineages are present.

By "human peripheral blood cells originating from human bone marrow" is meant cells circulating in the peripheral blood such as mononuclear cells or $CD34^+$ cells that were released into the blood stream and had their origin in bone marrow.

By "sorted human bone marrow cells" is meant human bone marrow cells that have undergone a separation process whereby cell surface markers or cell determinants are used to separate mixtures of cells into different populations. Sorting can be via FACS (Fluorescent Activated Cell Sorter) or magnetic separation using antibody cocktails, microbeads and magnets. Sorting can be positive selection for the cells of interest (e.g., $CD34^+$ cells, which are selected with α-CD 34 antibodies to isolate cells expressing the CD34 antigen) or negative selection whereby undesirable cells are removed from the population (e.g., use of a CD38 antibody to remove $CD38^+$ cells from a given population).

Unsorted and sorted murine bone marrow cells are as described for human cells except that the relevant murine surface antigen applies.

By "fetal liver cells" is meant cells that have migrated from the blood islands of the yolk sac to the fetal liver which develop into hematopoietic cells including embryonic

erythroid cells, macrophages and stem/progenitor cells. They are obtained as described in Jordan, C.T., McKearn, J.P., Lemischka, I.R. *Cell* 61:953-963, 1990. In addition, one could use a specific antibody, AA4.1, to enrich for a subpopulation of fetal liver tissue that includes multipotential stem/progenitor cells (Jordan, C.T., McKearn, J.P., Lemischka, I.R. *Cell* 61:953-963, 1990). Such cells have as surface markers CD34 and AC133.

By "yolk sac cells" is meant cells which are the origin of the first hematopoietic cells in a developing organism. Cells within the extraembryonic tissue in yolk sac migrate from blood islands of the yolk sac to the fetal liver where they build up to a large population of hematopoietic cells including embryonic erythroid cells, macrophages, stem cells, and progenitor cells.

By "human or murine embryonal carcinoma cells or lines" is meant pluripotent cells that can be derived from teratocarcinomas or by direct culture of normal embryos that can be cultured *in vitro*, are pluripotent, and can be induced to differentiate in presence of various inducing agents.

By "human or mouse pluripotential teratocarcinoma cells or lines" is meant transplantable cancer cells derived from teratoma, a disorganized mass of cells containing many varieties of differentiated tissue, mixed with undifferentiated stem cells that continue to divide and generate more of the differentiate tissues. Teratocarcinoma cells can be grown in culture as permanent cell lines and in a suitable medium they will continue to proliferate indefinitely without differentiating. However, these cells are multipotential and can be induced to undergo multilineage differentiation. If the medium is changed by adding an inducer of differentiation, such as retinoic acid, or if the cells are allowed to aggregate, the cells can be triggered to differentiate into a variety of apparently normal specialized cell types.

By "murine pluripotent embryonic cells" is meant cells derived from murine blastocysts that when injected into blastocysts, the cells can colonize the germline and reconstitute a mouse.

By "human embryonic stem (ES) cell lines" is meant cell lines derived from early mammalian embryo that are totipotent and capable of *in vitro* proliferation (Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swwiergiel, J.J., Marshal, V.S., Jones, J.M.,

Science 282: 1145-1147, 1998; Bongso, A., C.Y. Fong, S.C. Ng, and S. Ratnam, *Hum. Reprod.* 9: 2110, 1994).

By "cell derived from the murine AGM" is meant cells derived from an area comprising dorsal aorta, gonads and mesonephros in mammalian embryos that contains a high level of hematopoietic stem/progenitor cells during murine embryogenesis (Medvinsky, A.L., Samoylina, N.L., Muller, A.M., Dzierzak, E.A., *Nature* 364:64-67, 1993; Medvinsky, A.L., Gan, O.I., Semenova, M.L., Samoylina, N.L., *Blood* 87:557-566, 1996).

By "cells involved in organ or tissue regeneration" is meant cells that divide by duplication such as kidney cells, endothelial cells that form the lining of blood vessels, and liver cells (Michalopoulous, G.K., and DeFrances, M.C. *Science* 276:60-66, 1997), as well as other cell populations that are renewed by means of stem cells, such as epidermis, bone, skeletal muscle, and nervous tissue (McKay, R., Science 276:66-71, 1997).

By "hematopoietic cells" is meant blood forming cells.

By "cells of neural origin" is meant cells that originate from ectoderm and develop into neural tube or neural crest to ultimately form the central nervous system and the peripheral nervous system.

By "human bone marrow cells that have undergone RBC lysis"is meant whole bone marrow aspirates that were depleted of the majority of red blood cells (RBCs) and platelets through RBC lysis and washes. This population is expected to include all other hematopoietic lineages.

By "human bone mononuclear cells" is meant cells in circulating blood and bone marrow that contain a single nucleus such as monocytes, lymphocytes, and NK cells. These cells can be isolated from the remaining red blood cells, platelets, and granulocytes on Histopaque 1077 solution of polysucrose and sodium diatrizoate adjusted to a density of 1.077 g/m (SIGMA Diagnostics, St. Louis, MO) or a similar product.

By "human bone marrow $CD34^+$ cells" is meant a collection of stem/progenitor cells derived from human bone marrow that express the CD34 surface marker on the cell surface. Typically, these cells are isolated using magnetic or FAC sorting using antibodies specific for the CD34 molecule.

By "FDCP-mix murine hematopoietic stem cell line" is meant murine cell lines that were cloned and isolated from long-term murine bone-marrow cultures infected with a recombinant of the Molony murine leukemia virus and the src oncogene of the Rous sarcoma virus (Spooncer, E., Heyworth, C.M., Dunn, A., Dexter, T. M. *Differentiation* 31: 111-118, 1986). These cell lines have many of the characteristics of hematopoietic stem cells.

By "B6SUTA murine hematopoietic stem cell line" is meant a factor-dependent hematopoietic cell line established from nonadherent cell populations removed from continuous mouse bone marrow cultures (Greenberger, J.S., Sakakeeny, M.A., Humphries, R.K., Eaves, C.J. and Eckner, R.J., *Proc. Natl. Acad. Sci. USA* 80:2931-2935, 1983).

By "P19 teratocarcinoma cells" is meant a cell line initiated by McBurney, et al, (McBurney, M.W. and Rogers, B.J., *Developmental Biology* 89: 503-508, 1982) that is a teratocarcinoma cell line derived from an embryonal carcinoma induced in a C3H/He strain mouse.

By "NTera-2 pluripotential embryonal carcinoma cells" is meant a pluripotent human embryonal carcinoma cell line which was derived from a single cell clone of NTera-2 cells which were established from a nude mouse xenograft tumor of Tera-2 cells (Andrews, P.W., Damjanov, I., Simon, D., Banting, G.S., Carlin, C., Dracopoli, N.C. Fogh, J., *Laboratory Investigation* 50:147-162, 1984). The Tera-2 cells were isolated from a lung metastasis from a human male with primary embryonal carcinoma of the testis.

In another preferred embodiment, the invention features a method to identify agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with human bone marrow cells having surface molecules comprising the growth factor receptors, harvesting primary and/or secondary lymphoid organs from the animals and isolating RNA from the organs, creating a library from the RNA, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, and screening in a functional assay the scFv fragments that bind the cell surface molecules to identify those that are agonist antibodies for the growth factor receptors.

By "harvesting" is meant sacrificing the animals and collecting primary and/or secondary lymphoid organs such as blood, spleen and bone marrow.

By "primary and secondary lymphoid organs" is meant to include but is not limited to organs such as blood, bone marrow, spleen, and lymph nodes.

By "isolating RNA" is meant lysing cells in the presence of agents that inhibit RNase activity, such as phenol and guanidine thiocyanate (Molecular Research Center, Inc., Cincinnati, OH) and separating RNA from DNA and proteins by centrifugation or other methods known to those who practice the art.

In further preferred embodiments, the stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

In another preferred embodiment, the invention features a method to identify agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with human bone marrow cells having surface molecules comprising the growth factor receptors, harvesting primary or secondary lymphoid organs from the animals and isolating RNA from the organs, creating a library from the RNA, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing the Fab fragments that bind the cell surface molecules, and screening in a functional assay the dimerized Fab fragments to identify those that are agonist antibodies for the growth factor receptors.

In further preferred embodiments, the stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

The invention also features a method to make agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the antibodies are surface displayed, screening using target cells the antibodies that are surface displayed to identify antibodies that are agonist antibodies for the growth factor receptors cells, and synthesizing the agonist antibodies.

By "synthesizing the agonist antibodies" is meant introducing the nucleic acid encoding the agonist antibody into a cell for the synthesis of the antibody and preferably its secretion into extracellular medium such that it can be isolated. High level expression of heterologous proteins has been achieved in many different systems including *E. coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*, mammalian cells, plants, and insect cells. Engineered antibody fragments have been expressed in many of these organisms including bacteria (Pluckthun, A., *Nature* 347: 497-498, 1990), yeast (Carlson, J.R. and Weissman, I.L., *Mol. Cell. Biol.* 8:2647-2650, 1988), plants (Hiatt, A., Cafferkey, R. Bowdish, K., *Nature* 342: 76-78, 1989), and mammalian cells. The incorporation of a molecular tag such as HIS6 allows rapid and efficient purification through nickel-chelate chromatography from the

medium or from cell lysates (Kroiher, M., Raffioni, S., Steele, R.E., *Biochim. Biophys. Acta.* 1250: 29-34, 1995; Burks, E.A. and Iverson, B.L., *Biotechnol. Prog.* 11: 112-114, 1995). Those who practice the art are familiar with these and other methods for synthesis of heterologous proteins and could readily adapt those methods to the synthesis of antibodies.

In another preferred embodiment, the invention features a method to make agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding the antibodies, cloning the nucleic acid sequences from the library into viral display vectors so that the antibodies are displayed on the surface of virus, screening using target cells the antibodies displayed on the surface of the virus for antibodies that are agonist antibodies to the growth factor receptors and synthesizing the agonist antibodies..

In a further preferred embodiment, the viral display vector is a bacteriophage vector or a phagemid vector.

In another preferred embodiment, the invention features a method to make agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, screening in a functional assay the scFv fragments that bind the cell surface molecules to identify those that are agonist antibodies for the growth factor receptors and synthesizing the agonist antibodies.

In another preferred embodiment, the invention features a method to make agonist antibodies to growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface

molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing the Fab fragments that bind the cell surface molecules, and screening in a functional assay the dimerized Fab fragments to identify those that are agonist antibodies for the growth factor receptors and synthesizing the agonist antibodies.

In further preferred embodiments, in the methods to identify and to make agonist antibodies to growth factor receptors the library is a combinatorial library; the target cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells; the screening of the scFv fragments is by bioassay for proliferation, differentiation or activation of target cells; the screening of the scFv fragments is by assaying for changes in transcription of downstream genes or phosphorylation of the receptor; the screening of the dimerized Fab fragments is by bioassay for proliferation, differentiation or activation of target cells; the screening of the dimerized Fab fragments is by assaying for changes in transcription of downstream genes or phosphorylation of the receptor.

By "combinatorial library" is meant a collection of nucleic acid molecules representing the immune repertoire of an organism where rearranged heavy and light chain V genes are combined at random creating many artificial V gene combinations. This significantly expands the number of antibody molecules that can be screened. Combinatorial libraries can be constructed as described in "Phage Display of Peptides and Proteins" Eds:

Brian K. Kay, Jill Winter, and John McCafferty, Chapter 6: "Construction and Screening of Antibody Display Libraries" by John McCafferty and Kevin S. Johnson. Academic Press, San Diego, CA 1996. The combinatorial libraries of the present invention represent a collection of nucleic acid sequences encoding the immune repertoire of an organism which contains nucleic acid sequences encoding antibodies or fragments thereof directed to surface molecules on the particular cell type utilized for immunization. The rearrangement of heavy and light chain V genes serves to simply expand the number of antibody molecules directed to these surface molecules, as artificial V gene combinations are produced, including those directed to cell surface molecules. This organized collection of nucleic acids has increased use for isolating antibody molecules or fragments thereof that specifically bind to receptors linked to a signal transduction cascade that leads to enhancement or inhibition of cellular proliferation, differentiation, activation or survival, e.g. growth factor receptors, displayed on the cell surface of the cell type utilized for immunization.

By "bioassay for proliferation or differentiation" is meant to also include cell survival and activation. For example such assays include, but are not limited to measuring Brdu incorporation, tritiated thymidine incorporation, changes in cellular enzyme levels such as mitochondrial dehydrogenase, visible colony formation, pH change, Ca^{++} concentration change and changes in gene transcription.

By "assaying for changes in the transcription of downstream genes" is meant assaying for changes in transcription of any gene that is regulated as part of a signal transduction cascade, e.g., nuclear transcription factors such as c-myc, c-jun, NF-κβ and c-fos. For example, exposing starved (serum and growth factor deprived) target cells to candidate antibodies for a brief period of time (e.g., 2 hrs) and isolating RNA from the cells. Changes in transcription can be detected (quantified) by methods that include RT-PCR using primers specific for one or more downstream genes, followed by agarose gel electrophoresis or by hybridizing RNA to silicon wafers having bound probes for specific genes. By utilizing such chips many genes can be assayed for simultaneously.

By "assaying for phosphorylation of the receptor" is meant the immunoprecipitation of the receptor with the antibody and then providing γ-ATP and examining phosphorylated products by SDS-PAGE or high-throughput screens for phosphorylation of the receptor.

The invention also features a method to identify growth factor receptors comprising the steps of generating agonist antibody to the receptors, and using the agonist antibody to identify the receptors.

Once an agonist antibody is identified, it can be used to identify the receptor to which it is binding. Those of ordinary skill in the art are familiar with techniques such as immunoprecipitation and/or immunoaffinity purification (Springer, T.A. (1997), "Isolation of Proteins Using Antibodies," *In* Current Protocols in Immunology (J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, eds.) pp. 821-829, John Wiley & Sons, New York) which are useful for the identification of the receptor.

In another preferred embodiment, the invention features a method to identify growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the scFv fragments are surface displayed, screening using target cells the surface displayed scFv fragments to identify those that are agonist antibodies for the growth factor receptors and using the agonist antibodies to identify the receptors.

In a further preferred embodiment the surface display vector is a bacteriophage vector and surface display is on the surface of bacteriophage.

In another preferred embodiment, the invention features a method to identify growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, screening in a functional assay said scFv fragments that bind the cell surface

molecules to identify those that are agonist antibodies for the growth factor receptors, and using the agonist antibody to identify the receptors.

In another preferred embodiment, the invention features a method to identify growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the scFv fragments are surface displayed, panning the surface displayed scFv fragments for binding to cell surface molecules on target cells, screening in a functional assay said scFv fragments that bind the cell surface molecules to identify those that are agonist antibodies for the growth factor receptors, and using the agonist antibody to identify the receptors.

In another preferred embodiment, the invention features a method to identify growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into cell surface display vectors so that the Fab fragments are surface displayed, panning the surface displayed Fab fragments for binding to cell surface molecules on target cells, dimerizing the Fab fragments that bind the cell surface molecules, screening in a functional assay the dimerized Fab fragments to identify those that are agonist antibodies for the growth factor receptors and using the agonist antibody to identify the receptors.

In a further preferred embodiments the surface display vector is a bacteriophage vector and surface display is on the surface of bacteriophage.

In another preferred embodiment, the invention features a method to identify growth factor receptors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library

from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing the Fab fragments that bind the cell surface molecules, screening in a functional assay the dimerized Fab fragments to identify those that are agonist antibodies for the growth factor receptors and using the agonist antibody to identify the receptors.

In a further preferred embodiment the growth factor receptor is a hematopoietic growth factor receptor.

By "hematopoietic growth factor receptor" is meant a receptor expressed on cells of hematopoietic origin that participates in the proliferation, differentiation, cell survival or functional activation of stem/progenitor or mature blood cells.

The invention further features a method to identify growth factors comprising the steps of generating an agonist antibody to growth factor receptor, using the agonist antibody to identify the receptor, and using the receptor to identify the growth factor.

There are a number of ways in which the native growth factor could be identified by using the growth factor receptor. For example, it is possible to transform cells with the cloned receptor under an inducible promoter for expression, followed by screening cDNA pools for effects in bioassay. For example, there is the use of the Cytosensor Microphysiometer System (Molecular Devices, Sunnyvale, CA), a biosensor to monitor receptor-mediated responses without previous knowledge of the signal transduction pathway. Alternatively, it is possible to make chimeric receptors to set up a well defined bioassay, such as increases in specific reporter gene expression. For example, the extracellular domain of the new receptor could be fused to the intracellular domain of a well characterized receptor, especially one in which downstream signaling events have been defined. Then, cDNA pools could be screened in bioassay for reporter gene expression. Those of ordinary skill in the art are familiar with these and other useful techniques.

In another preferred embodiment, the invention features a method to identify growth factors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of

immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the scFv fragments are surface displayed, screening the surface displayed scFv fragments to identify those that are agonist antibodies for the growth factor receptors using the agonist antibody to identify the receptor, and using the receptors to identify the growth factor.

In a further preferred embodiment, the surface display vector is a bacteriophage vector and surface display is on the surface of bacteriophage.

In another preferred embodiment, the invention features a method to identify growth factors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, and screening in a functional assay the scFv fragments that bind the cell surface molecules to identify those that are agonist antibodies for the growth factor receptors, using the agonist antibodies to identify the receptor, and using the receptors to identify the growth factor.

In another preferred embodiment, the invention features a method to identify growth factors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the Fab fragments are surface displayed, panning the surface displayed Fab fragments for binding to cell surface molecules on target cells, dimerizing the Fab fragments that bind the cell surface molecules, screening in a functional assay the dimerized Fab fragments to identify those that are agonist antibodies for the growth factor receptors

using the agonist antibody to identify the receptor, and using the receptors to identify the growth factor.

In a further preferred embodiment, the surface display vector is a bacteriophage vector and surface display is on the surface of bacteriophage.

In another preferred embodiment, the invention features a method to identify growth factors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing the Fab fragments that bind the cell surface molecules, screening in a functional assay the dimerized Fab fragments to identify those that are agonist antibodies for the growth factor receptors, using the agonist antibodies to identify the receptor, and using the receptors to identify the growth factor.

In further preferred embodiments the growth factor is a hematopoietic growth factor, the growth factor effects proliferation, differentiation, activation or survival of the following cell types: unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

The invention also features a method for screening for agonist antibodies comprising the steps of growing cells expressing antibody fragments in the presence of target cells

expressing receptors to which the antibodies are directed, and screening the antibody fragments to identify those that are agonist antibodies.

By "cells expressing antibody fragments" is meant prokaryotic or eukaryotic cells that carry cloned copies of antibody fragments linked to promoters and signal sequences specific to the host cell to enable cell surface antibody display, that may include transmembrane dimerization domains (Lemmon, M.A., Treutlein, H.R., Adams, P.D., Brunger, A.T., Engelman, D.M. *Nat. Struct. Biol.* 1:157-163, 1994), or to enable secretion of monomers and dimers of the antibody fragments.

By "in the presence of target cells" is meant coculturing cells expressing antibodies or fragments and cells expressing a receptor to which the antibody binds.

Screening involves other bioassays or biochemical assays to determine if the antibody fragment stimulates proliferation, differentiation, activation or survival of the target cell.

In preferred embodiments the cells expressing antibody fragments are bacterial cells, mammalian cells or yeast.

By "bacterial cell" is meant any prokaryotic cell, usually *E. coli* of any genotype or bacterial cells producing cell surface displayed molecules

By "mammalian cell" is meant any cell derived from mammalian origin, usually clonal with ability to grow *in vitro*.

By "yeast" is meant yeast cells, typically *Saccharomyces cerevisiae*, *Schitzosacchamyces pombe*, or *Pichia pastoris* that allows for the expression of plasmids or integrated DNA engineered to produce antibody molecules, which are surface displayed or secreted.

In another embodiment, the invention features agonist antibodies to growth factor receptors produced by immunizing an animal with stem/progenitor cells.

In preferred embodiments the stem/progenitor cells are selected from the group unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem

(ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

In another preferred embodiment, the invention features agonist antibodies to growth factor receptors produced by the methods of the present invention.

The invention also features combinatorial libraries produced from immune cells generated by immunizing an animal with stem/progenitor cells. In another embodiment the invention features combinatorial libraries encoding antibody molecules or fragments thereof comprising nucleic acid sequences from immune cells of animals immunized with stem/progenitor cells. In a further embodiment, the invention features combinatorial antibody fragment libraries directed to surface molecules on stem/progenitor cells. In a still further embodiment the invention features a combinatorial library produced by immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cell proliferation, differentiation, survival or activation so as to generate a plurality of immune cells expressing one or more antibodies or antibody fragments thereof of the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding the antibodies or antibody fragments thereof, cloning the nucleic acid sequences from the library into surface display vectors so that the antibodies are surface displayed. In a preferred embodiment the receptor is a growth factor receptor.

In still another embodiment, the invention features a method of producing a combinatorial antibody library encoding antibodies or fragments thereof to receptors involved in cell proliferation, differentiation, survival or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising the receptors, so as to generate a plurality of immune cells expressing one or more antibodies or antibody fragments to the surface molecules, obtaining nucleic acid sequences encoding variable and constant regions of the antibody fragments, from the plurality of immune cells, randomly combining nucleic acid sequences encoding variable regions of the antibody fragments to produce a combinatorial library of nucleic acid sequences encoding the antibody fragments,

and cloning the nucleic acid sequences of the library into surface display vectors so that the antibody fragments are surface displayed. In a preferred embodiment the receptor is a growth factor receptor.

By "randomly combining" is meant combining nucleic acid sequences encoding rearranged heavy and light chain variable genes to create many artificial variable gene combinations, e.g., by using a PCR overlap reaction.

In preferred embodiments, the stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.; the animal is a rabbit or a chicken; the antibody or fragments thereof is a scFv fragment; the antibody or fragments thereof is a Fab fragment; the antibodies or fragments thereof encoded by the combinatorial library are surface displayed on phagemeids; the method of producing further includes the step of screening serum obtained from the immunized animal for binding to the stem/progenitor cell, prior to obtaining nucleic acid sequences encoding the antibody fragments.

The invention also features various equivalent aspects directed toward the identification, synthesis and use of antibodies that are inhibitory to the proliferation, differentiation, activation or survival of cells. These aspects are similar to those concerning agonist antibodies and one of ordinary skill in the art would be able to adapt these so as to apply to inhibitory antibodies.

Furthermore, the invention features a method to identify inhibitory antibodies involved in cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having receptors involved in cellular

proliferation, differentiation or activation so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the antibodies are surface displayed, and screening the surface displayed antibodies for those that inhibit proliferation, differentiation or activation of target cells, so as to identify inhibitory antibodies.

By "inhibitory antibody" is meant entire antibodies or fragments thereof, including Fab fragments and scFv fragments, etc., that bind to a cell surface receptor that is linked to a signal transduction cascade that is involved in cellular proliferation, differentiation, activation, inhibition or survival and inhibits the proliferation, differentiation, activation or survival of a cell. Inhibitory antibodies could inhibit by blocking the action of a receptor that promotes proliferation, differentiation, activation or survival of the cell or by mimicking an inhibitory molecule that binds to a receptor that sends a negative growth signal.

By "proliferation" is meant active division and progression through the various stages of the cell cycle as detected by changes in the rate of protein synthesis, chromosome replication, cell size or cell number.

By "differentiation" is meant changes in cell morphology, behavior or function that lead to the production of different types of cells with specialized functions, as a result of exposure to extrinsic factors, or changes in gene expression. Differentiation refers to the process by which cells mature and become less pluripotent, as that term is used in the art.

By "activation" is meant the process by which cells leave G_0 and enter G_1, but do not synthesize DNA or divide until a second signal is received. Activation is associated with the expression of a specific set of activation genes and activation antigens.

By "survival" is meant that cells do not undergo apoptosis or programmed cell death or necrosis.

Receptors involved in proliferation, differentiation, activation or survival could represent the same receptors that bind agonist antibodies if the inhibitory antibody is acting as an antagonist or blocking antibody. The receptor may be different from the growth factor receptor that agonist antibodies bind if the inhibitory antibody is mimicking an inhibitory factor.

In another preferred embodiment, the invention features a method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding the antibodies, cloning the nucleic acid sequences from the library into viral display vectors so that the antibodies are displayed on the surface of virus, and screening the antibodies displayed on the surface of the virus for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

In a further preferred embodiment the viral vector is selected from the group consisting of bacteriophage and phagemid vectors.

In another preferred embodiment, the invention features a method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, and screening the scFv fragments displayed on the surface of the phagemid for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

In another preferred embodiment, the invention features a method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from

the library into surface display vectors so that the scFv fragments are surface displayed, and screening the surface displayed scFv fragments for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

In a further preferred embodiment the surface display vector is a bacteriophage vector and surface display is on the surface of bacteriophage.

In another preferred embodiment, the invention features a method to identity inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/ progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, and screening in a functional assay the scFv fragments that bind the cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

Panning and screening are carried out as previously described.

In another preferred embodiment, the invention features a method to identity inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/ progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the scFv fragments are surface displayed, panning the surface displayed scFv fragments for binding to cell surface molecules on target cells, and screening in a functional assay the scFv fragments that bind the cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

In a further preferred embodiment the surface display vector is a bacteriophage vector and surface display is on the surface of bacteriophage.

In another preferred embodiment the invention features a method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing Fab fragments the bind cell surface molecules, and screening in a functional assay as both monomer and dimerized the Fab fragments that bind the cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

Panning and screening are carried out as with scFv fragments.

By "dimerizing Fab fragments" is meant that a representative portion of the Fab fragments that bind to cell surface molecules are dimerized. The remainder of the Fab fragments are left as monomers. Both monomers and dimers are tested in the functional assays.

By "screening in a functional assay as both monomer and dimerized Fab fragments" is meant that monomers and dimers are independently screened (see discussion on scFV fragments). In addition the Fab fragments (monomers and dimers) may be either surface displayed or soluble molecules.

In another preferred embodiment, the invention features a method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid

sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the Fab fragments are surface displayed, panning the surface displayed Fab fragments for binding to cell surface molecules on target cells, dimerizing Fab fragments that bind said cell surface molecules, and screening in a functional assay both monomers and dimerized Fab fragments bind to cell surface molecules on target cells for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

In a further preferred embodiment the surface display vector is a bacteriophage vector and surface display is on the surface of bacteriophage.

In still further preferred embodiments of the methods to identify inhibitory antibodies the animal is a rabbit or a chicken; the stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

In another preferred embodiment the invention features a method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation differentiation or activation, harvesting primary or secondary lymphoid organs from the animals and isolating RNA from the organs, creating a library from the RNA, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, and screening

in a functional assay the scFv fragments that bind the cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

In a further preferred embodiment the stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

In another preferred embodiment the invention features a method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation differentiation or activation, harvesting primary or secondary lymphoid organs from the animals and isolating RNA from the organs, creating a library from the RNA, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing Fab fragments that bind said cell surface molecules, and screening in a functional assay both monomers and dimerized Fab fragments that bind said cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

In a further preferred embodiment the stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone

marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

The invention also features a method to make inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the antibodies are surface displayed, screening the surface displayed antibodies for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, and synthesizing the inhibitory antibodies.

Synthesis of inhibitory antibodies is carried out as previously described for agonist antibodies.

In another preferred embodiment, the invention features a method to make inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding the antibodies, cloning the nucleic acid sequences from the library into viral display vectors so that the antibodies are displayed on the surface of virus, screening the antibodies displayed on the surface of the virus for those that inhibit growth, proliferation, differentiation or

activation of target cells so as to identify inhibitory antibodies, and synthesizing the inhibitory antibodies.

In a further preferred embodiment the viral vector is a bacteriophage vector or a phagemid vector.

In another preferred embodiment the invention features a method to make inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, screening by functional assay the scFv fragments that bind the cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, and synthesizing the inhibitory antibodies.

In another preferred embodiment the invention features a method to make inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem or progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning said nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing Fab fragments that bind the cell surface molecules, and screening by functional assays both monomers and dimerized Fab fragments that bind the cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, and synthesizing the inhibitory antibodies.

In further preferred embodiments in the methods to identify inhibitory antibodies of claims the library is a combinatorial library; the target cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells; the screening by functional assay of the scFv fragments is by bioassay for the inhibition of proliferation, differentiation or activation of target cells; the screening by functional assay of the dimerized Fab fragments is by bioassay for the inhibition of proliferation, differentiation or activation of target cells.

In another embodiment the invention features a method to identify receptors involved in cellular proliferation, differentiation or activation comprising the steps of generating an inhibitory antibody to the receptors, and using the inhibitory antibody to identify the receptor.

Identification of the receptor using the inhibitory antibody to the receptor is carried out as previously described for agonist antibodies.

In another preferred embodiment, the invention features a method to identify receptors involved in cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the scFv fragments are surface displayed, screening the surface displayed scFv fragments for those that inhibit proliferation, differentiation or activation of

target cells so as to identify inhibitory antibodies, and using the inhibitory antibodies to identify the receptors.

In a further preferred embodiment, the surface display vector is a bacteriophage vector and surface display is on the surface of a bacteriophage.

In another preferred embodiment the invention features a method to identify receptors involved in cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, screening in a functional assay the scFv fragments that bind said cell surface molecules for those that inhibit proliferation, differentiation, or activation of target cells so as to identify inhibitory antibodies, and using the inhibitory antibody to identify the receptors.

In another preferred embodiment, the invention features a method to identify receptors involved in cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the Fab fragments are displayed on the surface of cells, panning the Fab fragments displayed on the surface of cells for binding to cell surface molecules on target cells, dimerizing Fab fragments that bind the cell surface molecules, screening in a functional assay both monomer and dimerized Fab fragments for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, and using the inhibitory antibody to identify said receptors.

In a further preferred embodiment, the surface display vector is a bacteriophage vector and surface display is on the surface of a bacteriophage.

In another preferred embodiment, the invention features a method to identify receptors involved in cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that the Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing Fab fragments that bind said cell surface molecules, screening in a functional assay both monomer and dimerized Fab fragments for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, and using the inhibitory antibody to identify the receptors.

In a further preferred embodiment of the methods to identify the receptor involved in cellular proliferation, differentiation or activation, the receptor is a hematopoietic receptor.

The invention further features a method to identify inhibitory factors to cellular proliferation, differentiation or activation comprising the steps of generating inhibitory antibodies to receptors involved in cellular proliferation, differentiation or activation, using the inhibitory antibody to identify the receptor, and using the receptors to identify the inhibitory factor.

The receptor is used to identify the inhibitory factor as previously described for identification of growth factors.

In another preferred embodiment, the invention features a method to identify inhibitory factors to cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv

fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the scFv fragments are surface displayed on the surface of cells, screening the surface displayed scFv fragments for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, using the inhibitory antibody to identify the receptor, and using the receptors to identify the inhibitory factor.

In a further preferred embodiment, the surface display vector is a bacteriophage vector and surface display is on the surface of a bacteriophage.

In another preferred embodiment, the invention features a method to identify inhibitory factors to cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that said scFv fragments are displayed on the surface of phagemid, panning the scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, and screening in a functional assay the scFv fragments that bind the cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, using the inhibitory antibodies to identify the receptor, and using the receptors to identify the inhibitory factor.

In another preferred embodiment, the invention features a method to identify inhibitory factors comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into surface display vectors so that the Fab fragments are surface displayed, panning the surface displayed Fab fragments for binding to cell surface molecules on target cells, dimerizing Fab fragments that bind the cell surface

molecules, screening in a functional assay both monomer and dimerized Fab fragments for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, using the inhibitory antibody to identify the receptor, and using the receptors to identify the inhibitory factor.

In a further preferred embodiment, the surface display vector is a bacteriophage vector and surface display is on the surface of a bacteriophage.

In another preferred embodiment, the invention features a method to identify inhibitory factors to cellular proliferation, differentiation or activation comprising the steps of immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to the surface molecules, creating a library from the plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of the antibodies, cloning the nucleic acid sequences from the library into phagemid vectors so that Fab fragments are displayed on the surface of phagemid, panning the Fab fragments displayed on phagemids for binding to cell surface molecules on target cells, dimerizing Fab fragments that bind the cell surface molecules, screening in a functional assay both monomer and the dimerized Fab fragments for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, using the inhibitory antibodies to identify the receptor, and using the receptors to identify the inhibitory factor.

In a further preferred embodiment of the methods to identify inhibitory factors, inhibitory factor is a hematopoietic factor.

In another embodiment, the invention features a method for screening for inhibitory antibodies for cellular proliferation, differentiation, activation or survival comprising the steps of growing cells expressing antibody fragments in the presence of target cells expressing receptors to which said antibodies are directed, and screening the antibody fragments for those that inhibit proliferation, differentiation, activation or survival of target cells so as to identify inhibitory antibodies.

In further preferred embodiments the cells expressing antibody fragments are selected from the group consisting of bacterial cells, mammalian cells and yeast.

Also, the invention features inhibitory antibodies to receptors involved in proliferation, differentiation, activation or survival made by immunizing an animal with stem/progenitor cells.

In preferred embodiments the stem/progenitor cells selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis. human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

In further preferred embodiments the invention features inhibitory antibodies to receptors involved in proliferation, differentiation, activation or survival made by any of the methods of making inhibitory antibodies.

In addition, the invention features a method for treating a patient having a disease or disorder characterized by a deficiency in a cell population comprising the step of administering to the patient a therapeutically effective amount of an agonist antibody produced by immunizing an animal with stem/progenitor cells that stimulates the proliferation or differentiation of cell of the population.

By "disease or disorder characterized by a deficiency in a cell population" is meant for example diseases, disorders or treatment related to suppression of hematopoiesis where less than the normal number of cells of a given lineage or lineages are present in a patient. For example, patients undergoing chemotherapy show subnormal levels of neutrophils and platelets. Anemias are examples of subnormal levels of red blood cells.

By "administering" is meant provision of an antibody.

By "therapeutically effective amount" is meant an amount which at least partially alleviates or abrogates some of the symptoms associated with the disease or condition.

In a preferred embodiment, the cell population is an hematopoietic cell population.

In another preferred embodiment, the invention features a method for treating a patient having a disease or disorder characterized by a deficiency in a cell population comprising the step of contacting *ex vivo* patient's cells with an agonist antibody produced by immunizing an animal with stem/progenitor cells that stimulates the proliferation or differentiation of the cells of the population.

By "ex vivo" is meant outside the body. Those of ordinary skill in the art are familiar with techniques for the isolation of cells from a patient, conditions for the maintenance of cells outside a patient and the reintroduction of cells into a patient.

In a preferred embodiment, the cell population is an hematopoietic cell population.

In another embodiment, the invention features a method for treating a patient having a disease or disorder characterized by an increase in a cell population comprising the step of administering to the patient a therapeutically effective amount of an inhibitory antibody produced by immunizing an animal with stem/progenitor cells that inhibits the proliferation or differentiation of the cell population.

By "disease or disorder characterized by an increase in a cell population" is meant diseases or disorders where greater than the normal number of cells of a given lineage or lineages are present in a patient. For example, chronic myeloid leukemia is a disease characterized by myeloid proliferation due the presence of a chromosomal translocation in the hematopoietic stem cells. Thrombocytopenia is a disease characterized by an increase in the numbers of megakaryocytes.

In a preferred embodiment, the cell population is an hematopoietic cell population.

In a further embodiment, the invention features a method for treating a patient having a disease or disorder characterized by cells exhibiting abnormal growth comprising the steps of administering to the patient an amount of an inhibitory antibody that inhibits the proliferation or differentiation of normal cells and that was produced by immunizing an animal with stem/progenitor cells, and killing the cells exhibiting abnormal growth.

By "cells exhibiting abnormal growth" is meant cells that do not exhibit growth control, such as cancer cells.

By "killing" is meant subjecting the cells that exhibit abnormal growth to an agent that results in cell death, such as radiation or a chemotherapeutic agents. Those of ordinary skill in the art are familiar with the administration of such agents to patients. Killing can be done *in vivo* or *ex vivo*.

In a preferred embodiment, the cells are hematopoietic cells.

In a further embodiment, the invention features a method for treating a patient having a disease or disorder characterized by cells exhibiting abnormal growth comprising the steps of administering to the patient an amount of an antibody that specifically binds to the cells and is linked to a toxin or radiolabel and that was produced by immunizing an animal with stem/progenitor cells, such that the antibody binds the cells exhibiting abnormal growth and results in their death.

The antibodies of the present invention can be coupled to a toxin protein to form a fusion immunotoxin that can be used to treat malignancies or other disease states. Toxic protein that can be linked to antibody binding domains include diphtheria toxin, Pseudomonas exotoxin A, among others (see Hertler, A.A., Frankel, A.E., *J. Clin. Oncol.* 7:1932-42, 1989). Those of skill in the art are familiar with the construction of such fusion immunotoxins.

The invention also provides a method for identifying cells comprising the step of contacting cells with an antibody specific for the cell type produced by immunizing an animal with stem/progenitor cells under conditions were the antibody specifically binds the cells, and detecting binding of the antibody.

By "specifically binds" is meant binds to an antigenic determinant is specific for a particular cell type.

In preferred embodiments, the cells are hematopoietic cells; the antibody is linked to a radiolabel.

Those of skill in the art are familiar with the use of antibodies as imaging agents (see Breitz, H.B., Tyler, A., Bjorn, M.J., Lesley, T., Weiden, P.L., *Clin. Nucl. Med.* 22:615-620, 1997 and Li Destri, G., Greco, S., Rinzivillo, C., Racalbuto, A., Curreri, R., Di Cataldo, A., *Surg. Today* 28:1233-1236, 1998).

In another embodiment, the invention features a method for isolating a population of specific cells comprising the step of contacting a sample potentially containing the specific

cells with an antibody that specifically binds to the population of cells produced by immunizing an animal with stem/progenitor cells under conditions were the antibody specifically binds the population of cells, and using the bound antibody to isolate the population of cells.

By "using the bound antibody to isolate" is meant using the identified antibodies in cell isolation strategies such as fluorescence-activated cell sorting (FACS), or magnetic sorting procedures. In FACS sorting, the antibody is often conjugated to fluorescent molecules. In magnetic sorting procedures, the antibody is linked directly or indirectly to magnetic microbeads.

In a preferred embodiment, the cells are hematopoietic cells.

In a further embodiment, the invention features a method for amplifying a population of cells comprising the step of contacting the population of cells with an agonist antibody that stimulates proliferation or differentiation of the cells and that was produced by immunizing an animal with stem/progenitor cells.

By "amplifying" is meant causing the proliferation of a cell or cells.

In a preferred embodiment, the cells are hematopoietic cells.

In another embodiment the invention provides a method of modulating activity of a receptor involved in cellular proliferation, differentiation, survival or activation which is present on the surface of a stem/progenitor cell by binding to the receptor an antibody or fragment thereof produced by immunizing an animal with the stem/progenitor cells.

By "modulating is meant stimulating, inhibiting, or blocking the activation of the receptor.

In a further preferred embodiment, the receptor is a growth factor receptor.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

All articles, publications and patents cited in this application are hereby incorporated by reference, in their entirety.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a diagrammatic representation of a vector used for phage display.

Figs. 2A and B are diagrammatic representations of the primers utilized in a scFv cloning scheme.

Figs. 3A, 3B, 3C and 3D are diagrammatic representations of the primers used in a Fab cloning scheme.

Fig. 4 is a diagrammatic representation of a ScFv cloning scheme.

Figs. 5A and 5B are diagrammatic representations of vectors used in a Fab cloning scheme.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following examples are provided for further illustrating various aspects and embodiments of the present invention and are in no way intended to be limiting in scope Those in the art would appreciate that the examples concerning hematopoietic cells are applicable to the screening of other factors that participate in the proliferation, differentiation, activation and survival of cells of other systems. In addition, those of ordinary skill in the art appreciate and would be readily able to adapt these methods to identify inhibitory antibodies, receptors and inhibitory factors.

Example 1: Screening for agonist antibodies for hematopoietic growth factor receptors

Immunization

Chickens and rabbits are immunized with each of the following separately: human bone marrow cells (sorted and unsorted) (Poietic Technologies, Germantown, MD), murine bone marrow cells(sorted and unsorted) (Rabbit & Rodent Diagnostic Associates, San Diego, CA), fetal liver cells (obtained as described in the Jordan, C.T., McKearn, J.P., Lemischka, I.R. *Cell* 61:953-963, 1990). In addition, one could use a specific antibody, AA4.1, to enrich for the stem cells, yolk sac cells (Rabbit & Rodent Diagnostic Associates, San Diego, CA), mononuclear cell preparations (Poietic Technologies, Germantown, MD). Immunization can also be with murine hematopoietic cell lines such as FDCP-Mix (Spooncer, E., Heyworth, C.M., Dunn. A., Dexter, T.M. *Differentiation* 31:111-118, 1986) and B6SUTA (Greenberger, J.S., Sakakeeny, M.A., Humphries, R.K., Eaves, C.J. Eckner, R.J. *Proc. Natl. Acad. Sci. USA* 80: 2931-2935, 1983). Also, hematopoietic stem/progenitor cells isolated

from circulating blood, as previously described. In addition, immunization can be with other cells such as P19 cells, NTera-2 cells and human ES cell lines. Sorting of bone marrow cells is carried out via magnetic sorting using antibodies (e.g.. α-CD 34) attached to magnetic beads. The cells are precoated with monoclonal antibodies that recognize cell surface molecules. The monoclonal antibodies are attached to magnetic beads through secondary antibodies conjugated to the beads. The cells are then removed with a magnet. Magnetic sorting can be positive selection where the cells of interest are bound by the antibody (e.g., stem cell populations bound to antibody) (Miltenyi Biotec, Auburn, CA) or negative sorting where undesired cells are captured by the magnet (Stem Cell Technologies, Vancouver BC, Canada).

In fluorescence activated sorting, cells tagged with antibodies conjugated to fluorescent molecules are sorted electronically on a flow cytometer such as Becton-Dickinson FACS IV or FACS vantage cytometer or an equivalent sorting machine. The fluorescent molecule conjugated antibodies recognize specific cell surface antigens. The antibodies are conjugated to fluorescent markers such as fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) (Becton-Dickinson, San Jose, CA; Biosource International, Camarillo, CA.).

Immunization can be by any acceptable route including but not limited to intraperitoneal, subcutaneous, intramuscular, intravenous. Immunizing, can be in the presence or absence of adjuvant. Generally 1-10 million cells are injected at primary immunization. Animals immunized broadly, such as with bone marrow preparations where RBCs are lysed and platelets are removed have all of the remaining lineages present, including stem/progenitor cells. By using these types of bone marrow preparations, the response to the antigen should be very broad with antibodies generated against a wide variety of surface receptors. Animals immunized with a sorted population, e.g. $CD34^+$ cells, will generate less diversity in the immune response, but may facilitate identification of an antibody with effects specific to stem cells or to one lineage.

Following primary immunization, animals are boosted, one or more times, with the same immunogens to generate secondary immune responses. Libraries generated from secondary immune responses should result in antibody fragments with higher affinities, due to affinity maturation.

At preimmunization and following booster immunizations, blood is collected and serum is prepared for evaluation of serum antibodies. Indirect cellular ELISAs with preimmune and immune serum against target cells are used to screen for antibodies against uncharacterized cellular antigens. In these whole cell ELISAs, target cells (1×10^7 cells/ml) are dispensed into microtiter plates (1×10^6 cells/well), and pelleted gently (1500rpm/4°C/5'). Culture supernatant are removed by aspiration. Cells are washed briefly in wash buffer (PBS/1% BSA/0.1% NaN_3) and pelleted. Cells are resuspended in 100 ul solutions containing serum dilutions at 1/50, 1/100, 1/500, 1/1000, 1/5000. Cells and antibodies are incubated 1 hour at 4°C. Samples are again pelleted gently. Supernatant is removed by aspiration and cells are washed twice in wash buffer. Pellets are resuspended in 100 ul of 2° antibody-enzyme conjugate (secondary goat anti-rabbit or goat anti-chicken antibody congugated to alkaline phosphatase or horse radish peroxidase). Plates are incubated at 4°C/1hr and cells are then washed twice in wash buffer. Pellets are resuspended in developer reagent, 100 ul/well. Developer is PNPP (Sigma, St. Louis, MO) for alkaline phosphatase secondary antibody or ABTS (Sigma, St. Louis, MO) for horseradish peroxidase antibody. The enzyme reaction is allowed to proceed and read visually or on microtiter plate reader at appropriate wavelength. Only immunizations that produce a positive serum ELISA test are carried to the next stage of the method (library construction).

Library construction

Within one week after final boost, following a positive serum ELISA test, animals are harvested and primary and secondary lymphoid organs (spleen, bone marrow, and blood) are rescued. Peripheral blood lymphocytes are isolated from blood on a Ficoll or Percol cushion (Sigma, St. Louis, MO). RNA is isolated from spleen, bone marrow, and peripheral blood lymphocytes by a phenol/guanidine thiocyanate procedure (Tri Reagent, Molecular Research Center, Cincinnati, OH). RNA is reverse transcribed to cDNA using oligo$(dT)_{15}$ primers, deoxynucleotides, and AMV Reverse Transcriptase using a first strand cDNA synthesis kit (Boehringer Mannheim, Indianapolis, IN).

Fab fragment and/or single chain variable region (scFv) expression libraries are constructed from each immunized animal. The surface display vector for each library is pRL4 (see Figure 1), which enables display of chimeric expression products on the surface of

packaged phagemid particles. pRL4 is a modified version of pComb3H (Barbas, C.F. III and Burton, D. R. 1994. Monoclonal Antibodies from Combinatorial Libraries. Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor, N.Y.; Burton, D.R.; Barbas, C.F. III. *Advances in Immunology* 57:191-280, 1994; Lang, I.M., Chuang, T.L., Barbas, C.F. 3rd, Schleef, R.R. *J. Biol. Chem.* 271: 30126-30135, 1996) It was constructed by PCR amplification with primers NPCAMB-F1 (CACCATGGCGCATACCCGTACGACGTT CCGGACTACGCTTCTTAGGAGGGTGGTGGCTCT, SEQ. ID. NO. 1) and NPC3AMB-B (GCTTACAATTTCCCAGATCTGCG, SEQ. ID. NO. 2) and template plasmid pComb3H (Barbas, C.F. III and Burton, D.R., Cold Spring Laboratory Course Manual, Monoclonal Antibodies from Combinatorial Libraries" 1994). This amplifies a fragment containing hemagglutinin decapeptide (HA-dp) (Field, J., Nikawa, J.I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I.A., Lerner, R.A., Wigler, M. *Mol. Cell. Biol.* 8:2159-2165, 1988), an amber stop codon (TAG) and gene III. The resulting PCR product was used as the template in a second PCR reaction with primers NPC3AMB-F2 (GAGGAGGAGGAGGA GGAGACTAGTGGCCAGGCCGGCCAGCACCATCACCATCACCATGGCGCATACC CGT, SEQ. ID. NO. 3) and NPC3AMB-B (SEQ. ID. NO. 2), adding a HIS6 sequence and SfiI and SpeI restriction sites to the 5' end. The final PCR product was cloned into vector pComb 3H and confirmed by sequence analysis, and functional expression. In pRL4, antibody fragments are cloned into the SfiI restriction site.

In pRL4, the single chain antibody fragments are cloned downstream of the *E. coli* lacZ promoter, ribosome binding site, and *omp* A leader sequence. These elements allow induction of expression by IPTG, and secretion to the periplasm via the *Omp* A leader sequence.

In the final hybrid Fab expression construct in pRL4, the light and heavy chains are cloned as a single SfiI fragment. In this way, the light chain fragments are cloned downstream of the *E. coli* lacZ promoter, ribosome binding site, and *omp* A leader sequence. These elements allow induction of expression by IPTG, and secretion out of the cell via the *omp* A leader sequence. The light chain fragments are followed by sequences provided by the PCR primers and include a stop codon, a second ribosome binding site, and the *E. coli pel* B leader

sequence. Hybrid heavy chain genes are fused in frame with filamentous phage gene III (gIII) sequences (amino acids 230-406). An amber stop codon is present at the fusion junction. In a *sup E* bacterial host such as ER2357 (New England Biolabs, Beverly, MA), the amber mutation is suppressed. Upon promoter induction, a single polycistronic message is transcribed and translated as two polypeptides, a light chain and a heavy chain-gene III fusion protein. Following synthesis the polypeptides are transported to the bacterial periplasmic space as directed by the leader sequences. In the periplasmic space the heavy chain-pIII fusion proteins are inserted into the membrane, and the light and heavy chains are associated covalently through disulfide bonds, forming the antigen binding sites. The human constant region CH1 and C_L sequences include the cysteines that form the disulfide bond between heavy and light chains. Upon superinfection with helper phage, these fragments are exported out of the cell on the surface of phage as Fab-pIII fusions. In a non-*sup E* host, such as TOP10F' (Invitrogen, Carlsbad, CA), the amber stop codon is recognized yielding soluble Fab fragments. Other features of pRL4 include two molecular tags, HA and His6. The HA tag is recognized by HA.11 antibody (Babco, Berkeley, CA). The His6 tag allows affinity purification of antibody fragments by Nickel-chelate chromatography (Quiagen, Santa Carita, CA).

ScFv fragments

Single chain variable regions libraries (scFv)are constructed from each immunized animal. Single chain libraries are useful because the entire binding domain is contained on one polypeptide. The light chain variable region is separated from heavy chain variable region by a linker region. The use of short linkers (< 11 amino acids) favors a dimeric complex where V_H of one ScFv associates with V_L of another ScFv molecule and visa versa, these molecules are termed diabodies (Kortt, A.A., Malky, R.L., Caldwell, J.B., Gruen, L.C., Ivanci, N., Lawerence, M.G. et al. *Eur. J. Biochem.* 221:151-157, 1994). This is because folding of monomeric ScFv is impaired with linkers < 11 amino acids (Alfthan, K., Takkinen, K., Sizman, D., Soderlund, H., and Teeri, T.T. *Protein-Eng.* 8:725-731, 1995). Longer linkers (> 11 amino acids) favors folding of monomeric ScFv into a single antigen binding domain, thus precluding dimer formation. In the present example scFv fragments are constructed with short linkers, 7 amino acids in length, or long linkers, 18 amino acids in length.

The design of pRL4 allows for dimerization of scFv antigen binding domains on the phage surface and in soluble form as detailed below. When the plasmid is transformed into a *supE* bacterial host such as ER2537 (F' *Sup E,* New England Biolabs, Beverly, MA), the amber mutation is suppressed approximately fifty percent of the time. In this way half of the expressed scFvs are fused with the filamentous phage gene III protein (amino acids 230-406) and the other half will be terminated just prior to gene III to produce soluble scFv. Both the scFv-pIII fusion and soluble scFv products have the *Omp A* signal sequence and will be transported to the periplasm where they will be able to form dimeric scFv complexes, termed diabodies (Kortt, A.A., Malby, R.L., Caldwell, J.B., Gruen, L.C., Ivanci, N., Lawrence, M.C. et al. *Eur. J. Biochem.* 221: 151-157, 1994). Diabodies are expected to fold such that the V_H of one scFv will pair with the V_L of a second scFv-pIII resulting in divalent antibody fragments. Upon superinfection with helper phage, these diabodies are exported out of the cell on the surface of phage as pIII-antibody fragments. In a non-*sup E* host, such as TOP10F' (InVitrogen, Carlsbad, CA), the amber stop codon is recognized yielding soluble scFv diabodies.

Figure 2A & B is a diagrammatic representation of the primers utilized in a scFv cloning scheme. All immunoglobulin variable regions are composed of hypervariable regions, also known as complementarity determining regions (CDRs) proposed to form the antigen binding site, and less variable regions, known as framework regions (FR). Oligos are designed such that they will anneal to and amplify all framework regions of published antibodies of a given organism. Antibody sequences from many organisms have been compiled in a single source: Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., and Foeller, C. 1991. Sequences of Proteins of Immunological Interest. Vol. 1-3. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH Publication No. 91-3242. Sets of primers for amplification of the entire immunoglobulin repertoire of various animals have been developed and used successfully as described (Barbas, C.F. III, and Burton, D.R. 1994. *Monoclonal Antibodies from Combinatorial Libraries*. Cold Spring Harbor Laboratory Course Manual). These primers include restriction sites for cloning, and a linker sequence between the light and heavy chain variable regions that is used in PCR overlap extension, and to provide the amino acids to link the light and heavy chain

variable regions into a single polypeptide. Linker sequences can be short (for example coding for 7 amino acids) or long (for example coding for 18 amino acids). Rabbit variable light chain "forward" primers bind to a sequence within framework 1 (FR1) of a specific light chain family or families. Degeneracies are incorporated into the primers such that a single primer will amplify other closely related FR1 variable region sequences. Restriction sites are incorporated in the 5' end of each primer to facilitate cloning. Similarly, there are "reverse" primers for rabbit variable light chain sequences each annealing with a specific family or families of rabbit light chain FR4 regions. A restriction site and a linker sequence is incorporated in the 3' end of each of the "reverse" primers. For a single chain short library, the sequence is 5'-GGAAGAAGAGGAACC –3' (SEQ. ID. NO. 34). For the rabbit heavy chain variable region fragments, the sequences binding to FR regions are designed similarly, except primers are designed to anneal to published rabbit heavy chain sequences (Kabat, E.A., et al. 1991). A restriction site and linker sequence are incorporated in the 5' end of each of these primers for use during the second round of PCR amplification. The linker sequence incorporated in the heavy chain variable region primers is the reverse complement of the light chain linker sequence 5'-GGTGGTTCGTCTAGATCTTCC-3'(SEQ. ID. NO. 35). Briefly, variable heavy (V_H) and light (V_L) chains are amplified separately, then combined for an additional round of PCR amplification by overlap extension through the linker region to generate scFv products. This cloning step also permits random association of heavy and light chains. The entire products are amplified using oligos that bind to the extreme 5' and 3' ends of the resulting annealed products. The final PCR products, each containing one V_H region and one V_L region separated by a short linker(7 amino acids), is cloned into pRL4 (Figure 1). In order to construct scFv long libraries, the method is the same except that the sequence of the linker incorporated into the light chain reverse primers is extended by 33 nucleotides, coding for an additional 11 amino acids. In this case, the sequence CCCACCACCGCCC GAGCCACCGCCACCAGAGGA (SEQ. ID. NO. 36) is included just 3' of the nucleotides that encode the 7 amino acid short linker. The heavy chain primers for scFv long libraries are identical to those used in scFv short libraries. One of ordinary skill in the art can extrapolate from these designs and design other primers to amplify large immunoglobulin repertoire libraries from many different species.

Heavy and light chain variable region genes are amplified separately by PCR using cDNA as the template. Primers used will vary depending on the animal immunized. Construction of scFv libraries from rabbit is as follows (see Fig. 2): light chain variable region genes are amplified from first strand cDNA using forward Kappa primers RSCVK-1 (GGGCCCAGGCGGCCGAGCTCGTGMTGACCCAGACTCCA, SEQ. ID. NO.4), RSCVK-2 (GGGCCC AGGCGGCCGAGCTCGATMTGACCCAGACTCCA, SEQ. ID. NO. 5), RSCVK-3 (GGGCCCAGGCGGCCGAGCTCGTGATGACCCAGACTGAA, SEQ. ID. NO. 6), and forward lambda primer RSClambda1 RSCL-1 (GGGCCCAGGC GGCCGAGCTCGTGCTGACTCAGTCGCCCTC, SEQ. ID. NO. 7) (Gibco/BRL, Gaithersburg, MD) (see Fig. 2A). These primers bind to framework 1 (FR1) sequence of the different light chain families (Kabat, E.A. *et al.* 1991) and add restriction enzyme sites 5' of the variable sequences for cloning purposes. Reverse primers for Kappa light chain amplification are RKB9J0-B (GGAAGATCTAGAGGAACCACCTAGGATCTCCAGC TCGGTCCC, SEQ. ID. NO. 8), RKB9J10-B (GGAAGATCTAGAGGAACCACCTT TGATTTCCACATTGGTGCC, SEQ. ID. NO. 9) RKB42J0-B (GGAAGATCTAGAGG AACCACCTTTGACSACCACCTCGGTCCC, SEQ. ID. NO. 10), and reverse primer for lambda light chain amplification is Rjlambda0-B (GGAAGATCTAGAGGAACCAC CGCCTGTGACGGTCAGCTGGGTCCC, SEQ. ID. NO. 11) (Gibco/BRL, Gaithersburg, MD) (see Fig. 2A). These primers bind to FR4 sequences of the kappa and lambda light chain families, and include an extension sequence that is used to generate the linker sequence by PCR overlap extension. In the light chain reactions, each Kappa forward primer is paired with each Kappa reverse primer in nine separate reactions. The tenth light chain reaction uses the two lambda primers. Heavy chain variable region genes are amplified using forward primers RSCVH01 (GGTGGTTCCTCTAGATCTTCCCAGTCGGTGGAGGAGTCCRGG, SEQ. ID. NO. 12), RSCVH02 (GGTGGTTCC TCTAGATCTTCCCAGTCGGTGAAGGAGTCCGAG, SEQ. ID. NO. 13), RSCVH03 (GGTGGTTCCTCTAGATCTTCCCAGTCGYTGGAG GAGTCCGGG, SEQ. ID. NO. 14), and RSCVH04 (GGTGGTTCCTCTAGATC TTCCCAGSAGCAGCTGRTGGAGTCCGG, SEQ. ID. NO. 15) (Gibco/BRL, Gaithersburg, MD) (see Fig. 2B). These oligonucleotides bind to variable region framework 1 sequence and include a 5' extension used to generate linker sequence by overlap PCR extension. The

reverse primer for heavy chain amplification is RSCG-B (CCTGGCCGGCCTGGC CACTAGTGACTGAYGGAGCCTTAGGTTGCCC, SEQ. ID. NO. 16) (Gibco/BRL, Gaithersburg, MD) (see Fig. 2B). RSCG-B binds to rabbit constant FR4/CH1 sequence and carries an extension of restriction sites to facilitate cloning. In the heavy chain PCR reactions each forward primer is paired with the reverse primer for four separate heavy chain reactions.

Following heavy and light chain amplification and gel purification of fragments, all light chain and heavy chain fragments are combined and an additional round of PCR amplification allows for overlap extension. In this final overlap PCR reaction, light chain variable region fragments and heavy chain variable region fragments are annealed, and amplified. This PCR reaction uses primers RSC-F (GAGGAGGAGGAG GAGGAGGCGGGGCCCAGGCGGCCGAGCTC, SEQ. ID. NO. 17) (Gibco/BRL, Gaithersburg, MD) (see Fig. 2A) and RSC-B (GAGGAGGAGGAGGAGGAGCCT GGCCGGCCTGGCCACTAGTG, SEQ. ID. NO. 18) (Gibco/BRL, Gaithersburg, MD) (see Fig. 2B) which bind to restriction sites engineered onto light and heavy chain variable region sequences. This cloning step also permits random association of heavy and light chains. The PCR products are restricted with *Sfi* I. The final *Sfi* I restricted fragments, each containing one heavy chain variable region and one light chain variable region separated by a linker, is cloned into the *Sfi* I site of pRL4 (see Fig. 4). Ligation is transformed into *E. coli* ER2537 genotype: F'proA$^+$B$^+$lacI^q/Δ(lacZ)M15/ fhuA2(tonA)Δ(lac-proAB) supE thi-1Δ(hsdMS-mcrB)5 and ampicillin resistant (Amp^R) colonies are selected on a fraction of all the transformants. The remainder of the transformants are amplified in liquid culture. Colony counts are used to determine colony size. To confirm that antibody fragments have been successfully cloned in plasmids, random analysis of individual clones by restriction digest to check for appropriately sized inserts is performed. A single library of scFv fragments will contain greater than 10^7 individual members.

In the final single chain expression construct in pRL4, the single chain antibody fragments are cloned downstream of the *E. coli* lacZ promoter, ribosome binding site, and *omp* A leader sequence. These elements allow induction of expression by IPTG, and secretion out of the cell via the *omp* A leader sequence when expressed in the suppressor strain ER2537. The single chain fragments are fused in frame with filamentous phage gene III (gIII)

sequences (amino acids 230-406). The gIII protein product, pIII, is a minor coat protein necessary for infectivity. Upon promoter induction by IPTG, the single chain antibody-pIII fusion is synthesized and transported to the bacterial periplasmic space. In the periplasmic space, the scFv-gene III fusion proteins are inserted into the membrane. Upon superinfection with helper phage, these fragments are exported out of the cell on the surface of phage as pIII-antibody fragments. Other possible proteins to be used for fusion on the surface of phagemids include filamentous coat protein pVIII and other coat proteins.

For chicken scFv libraries, the method is essentially the same, but the primers used are specific to chicken light and heavy chain sequences (Kabat, E.A. *et al.*, 1991). The primer extension sequences and the linker sequences are the same as the rabbit primers to facilitate cloning into pRL4. Based on the exemplified primers and the sequences disclosed in Kabat, E.A. *et al.*, those of ordinary skill in the art could readily design primers for any species and construct scFv libraries.

Fab fragments

Fab fragment libraries, that maintain the native antigen recognition site, are useful to ensure that affinity is maintained.

Primers are designed based on published FR and constant region sequences (Kabat, E.A., et al. 1991). In the current scheme primers are used for hybrid antibody fragment construction (rabbit variable/human constant), however, primers can be designed similarly for constant regions from any species. For example, Fab fragment libraries derived from rabbit could have both variable and constant regions from rabbit. In this case, the variable region forward primers would remain the same, but reverse primers would contain constant region sequence from either rabbit kappa light chain, rabbit lambda light chain or rabbit heavy chain sequence. Hybrid Fab libraries can also be constructed. These libraries are composed of variable regions from immunized animals and constant regions (C_L and C_H1) from human IgG1. For hybrid Fab, each Fab fragment will be composed of variable regions from the immunized animals and constant regions (C_L and CH1) from human IgG1. Sets of primers for amplification of the immunoglobulin repertoire of rabbits and chickens, and for generation of chimeric Fabs, have been developed (Barbas, C.F. III, and Burton, D.R. 1994. *Monoclonal Antibodies from Combinatorial Libraries*. Cold Spring Harbor Laboratory Course Manual.

and November 1997 course on Monoclonal Antibodies from Combinatorial Libraries, Cold Spring Harbor Laboratory). "Forward" primers are paired with "reverse" primers to PCR amplify the rabbit variable kappa sequences, and one forward primer is paired with one reverse primer for lambda light chain variable region sequence amplification. The human constant kappa sequence is amplified from a phage display plasmid carrying a human antibody Fab (Persson, M.A., Caothien, R.H., and Burton, D.A. *Proc. Natl. Acad. Sci. U.S.A.* 88:2432-2436, 1991) using a "forward" primer that anneals to published human kappa constant region sequence (Kabat, E.A., et al. 1991), and a reverse primer that anneals to pelB leader sequence in the phage display plasmid. An overlap PCR reaction where all light chain fragments are pooled with the human constant kappa fragment generates hybrid light chain fragments. A similar approach is used for the heavy chain to generate hybrid heavy chain fragments. In the final and third round of PCR reactions, the hybrid light chains are pooled with the hybrid heavy chains and overlap PCR amplification is performed with external primers to generate fragments, each carrying both hybrid light chain and hybrid heavy chain sequences.

Heavy and light chain variable region genes are amplified by PCR using cDNA as the template. For rabbit Fab fragment libraries, construction is as follows: light chain variable region genes are amplified using the forward primers RSCVK1 (SEQ. ID. NO. 4), RSCVK2 (SEQ. ID. NO. 5), RSCVK3 (SEQ. ID. NO. 6) (Operon Technologies, Alameda, CA) (see Fig. 3A). These primers bind to framework 1 (FR1) sequence of the different Kappa light chain families (Kabat, E.A. *et al.*, 1991) and incorporate restriction enzyme sites 5' of the variable sequences for cloning purposes. Design and utilization of rabbit primers for the amplification of mRNAs encoding rabbit kappa light and gamma heavy chains for the construction of an antibody library from this species is described in: Lang, I.M., Barbas, C.F. 3rd, Schleef, R.R. *Gene* 172: 295-298, 1996. The lambda light chains are amplified with the forward primer RSCL-1 (SEQ. ID. NO. 7). Reverse primers for Kappa light chain amplification are RHHybK1-B (AGATGGTGCAGCCACAGTTCGTTTGATTT CCACATTGGTGCC, SEQ. ID. NO. 19), RHHybK2-B (AGATGGTGCAGCCAC AGTTCGTAGGATCTCCAGCTCGGTCCC, SEQ. ID. NO. 20), RHHybK3-B (AGATGGTGCAGCCACAGTTGCTTTGACSACCACCTCGGTCCC, SEQ. ID. NO. 21)

(Operon Technologies, Alameda, CA) (see Fig. 3A). The reverse primer for the lambda light chains is RHHybL-B (AGATGGTGCAGCCACAGTTCGGCCTGTGACGGT CAGCTGGGTCCC, SEQ. ID. NO. 22). These primers bind to FR4 sequence of the light chain families (Kabat, E.A. *et al.*, 1991), and include an extension of human constant region sequence. The extension enables human kappa chain constant region sequences to be added in a second round of PCR reactions. In the light chain reactions, each kappa forward primer is paired with each kappa reverse primer in nine separate PCR reactions. The tenth light chain PCR reaction uses the lambda forward and reverse primers. The human constant kappa sequences can be amplified from any human kappa chain clone. In this case, the forward primer, HKC-F (CGAACTGT GGCTGCACCATCTGTC, SEQ. ID. NO. 23) (see Fig. 3B) binds to human constant kappa sequence as published in Kabat, E.A. *et al.*, 1991. The reverse primer, lead B (GGCCATGGCTGGTTGGGCAGC, SEQ. ID. NO. 24), binds to plasmid sequences of a human kappa chain cloned into pComb3H. Alternatively, a primer may be designed to incorporate the sequences (e.g. the stop codon, the ribosome binding site, and the pelB leader sequence) necessary for expression in *E.coli*. Following gel purification of the fragments, a second PCR reaction is performed where all variable region PCR fragments are combined with the human constant kappa PCR fragments and primers RSC-F (SEQ. ID. NO. 17) (see Fig. 3A) and lead B (SEQ. ID. NO. 24). This reaction enables fusions of light chain variable regions to human constant kappa sequences.

Heavy chain variable region genes are amplified using the forward primers RhyVH1 (GCTGCCCAACCAGCCATGGCCCAGTCGGTGGAGGAGTCCRGG, SEQ. ID. NO. 25), RhyVH2 (GCTGCCCAACCAGCCATGGCCCAGTCG GTGAAGGAGTCCGAG, SEQ. ID. NO. 26), RhyVH3 (GCTGCCCAACCAGC CATGGCCCAGTCGYTGGAGGAGTCCGGG, SEQ. ID. NO. 27), and RhyVH4 (GCTGCCCAACCAGCCATGGCCCAGSAGCAG CTGRTGGAGTCCGG, SEQ. ID. NO. 28) as described in Lang, I.M., Barbas, C.F. 3rd, Schleef, R.R., *Gene* 172: 295-8, 1996 (see Fig. 3C). These primers include 5' sequences that encode the *pel* B leader sequence. The rabbit heavy chain reverse primer, RHHyIgGCH1-B (CGATGGGCCCTTGGTGGAGGCTGARGAGAYGGTGACCAGGGTGCC, SEQ. ID. NO. 29) binds to rabbit constant FR4 sequence and has an extension of human constant C_H1 sequence. Therefore, four separate PCR reactions are performed with each forward primer

and the reverse primer. The human constant IgG sequence can be amplified by PCR using a human antibody clone in pCOMB3H as the template and a forward primer that anneals to human constant IgG sequence, HIgGCH1-F (GCCTCCACCAAGGGCCCATCGGTC, SEQ. ID. NO. 30) and a reverse primer, dp-seq (AGAAGCGTAGTCCGGAACGTC, SEQ. ID. NO. 31) that anneals to plasmid sequences (Fig. 3). Alternatively, the human sequence can be amplified from any human antibody cDNA library using primers that anneal to constant region sequences as published in Kabat, E.A. *et al.*, 1991 and incorporate a restriction site on the reverse primer for cloning purposes. Following gel purification of the fragments, a second set of heavy chain PCR reactions is performed where all heavy chain variable region amplified fragments are combined with the human heavy chain constant region fragment. An overlap PCR extension reaction using primers lead VH (GCTGCCCAACCAGCCATGGCC, SEQ. ID. NO. 32) and dp-ex (GAGGAG GAGGAGGAGGAGAGAAGCGTAGTC CGGAACGTC, SEQ. ID. NO. 33) (see Fig. 3D) enables fusions of heavy chain variable regions to human constant region sequences. In the final overlap PCR reaction, hybrid light chains and hybrid heavy chains are annealed, and amplified using primers RSC-F (SEQ. ID. NO. 17) and dp-ex (SEQ. ID. NO. 33). The resulting fragment is restricted with appropriate enzymes, gel-purified and cloned as a single *SfiI* fragment containing both hybrid light and heavy chains into *SfiI* digested pRL4. Figure 5 is a diagrammatic representation of a Fab cloning scheme illustrating the final hybrid Fab fragments cloned into pRL4. Ligations are transformed into E. coli ER2537 and ampicillin resistant (Amp^R) colonies are selected on a fraction of all of the transformation. The remainder is amplified in liquid culture. Colony counts are used to determine library size.

For chicken Fab fragment libraries, the method is essentially the same, but the primers used are specific to chicken light and heavy chain sequences (Kabat, E.A. *et al.*, 1991). The primer extension sequences are the same as the rabbit primers to facilitate cloning into pRL4 and to generate the hybrid Fab fusions.

After libraries are constructed they are screened using target cells.

Target cells

Target cells are selected based on the immunogen that was used, the desired product, and the goal of the screen. For very broad range screens, it is important that the majority of

hematopoietic cell lineages are present in the immunizing population of cells. For example, immunizing with a population consisting of mononuclear cells, expected to include stem cells, progenitor cells, lymphocytes, monocytes, and NK cells would be fairly broad and has the potential to generate agonist antibodies or inhibitory antibodies to stem cells, progenitor cells of different lineages, lymphocytes, monocytes, or NK cells. An even broader screen would use whole bone marrow aspirates that were depleted of the majority of red blood cells (RBCs) and platelets through RBC lysis and washes. This population is expected to include all other hematopoietic lineages. Once these libraries are constructed, target cells for screening depends on the goal of the screen. To identify relevant antibodies for multiple lineages at once, the target cell population could be the same as the immunogen, a diverse population of cells, and likewise very broad. These studies will uncover interesting agonists and inhibitors against a wide variety of cells at once. For high throughput screening on a diverse population of cells it is necessary to confirm that each member of the antibody library is exposed to each cell lineage, at various stages of maturation. An alternative to this is to limit the target population to a single lineage to identify antibodies relevant to a single lineage. For example neutrophils can be isolated from cells of other lineages using light scattering signals in flow cytometry and a combination of the monoclonal antibodies, CD11b, CD15, and CD16 as described in: Terstappen, L.W., Safford, M., Loken, M.R. *Leukemia* 4: 657-663, 1990. Many other hematopoietic lineages can isolated for use as a target cell to identify relevant antibodies against a specific lineage.

Alternatively, one can identify relevant antibodies that play a role human hematopoietic stem cell renewal or differentiation. Studies suggest that the $CD34^+$ CD38- fraction of human bone marrow cells contains stem cells capable of long term engraftment and multilineage differentiation (reviewed in "Williams Hematology" Fifth Edition Editors: Ernest Beutler, Marshall A. Lichtman, Barry S. Coller, Thomas J. Kipps McGraw-Hill, U.S.A. in chapter "Hemopoietic stem cell, progenitor cells, and cytokines" by Peter J. Quesenberry). Because stem cells represent a very small fraction of human bone marrow cells (less than 1 in 1 X 10^5 bone marrow cells), ligands specific to the surface of primitive human stem cells could be identified from antibody libraries where human $CD34^+$ sorted primary human cells were used as immunogen. In this case, the target cells could be the $CD34^+$ population and

antibodies that recognize $CD34^+CD38^-$ cells could be identified by FACS sorting, magnetic sorting, or by panning on such target cells. Another target in this case could be murine hematopoietic stem cell lines such as FDCP-Mix or a murine multipotent hematopoietic stem cell line (Spooncer, E., Heyworth, C.M., Dunn. A., Dexter, T.M. 1986. *Differentiation* 31:111-118). The advantage of using a cell line as a target is its availability, clonal properties, and ease to work with.

Therefore, target cells are selected based upon criteria that include the immunogen that was used, the desired end product, the range of the screen desired, availability, ease to work with.

If the initial target cell is a murine cell, eventually testing will be carried out in the appropriate human cell line.

For hematopoiesis preferred primary cells and cell lines are lysed whole blood, human bone marrow mononuclear cells, FDCP-mix, and $CD34^+$ sorted bone marrow cells. For P19 cells the preferred target are p19 cells and for NTERA cells the preferred target cells are NTERA cells.

Panning

Phage (bacteriophage or phagemid) displayed antibody libraries are selected for phage carrying antibody fragments that bind to various target cells, and remain on the cell surface or are internalized, including mouse cell lines, human cell lines, and sorted and unsorted primary bone marrow samples, using a method termed panning, as well as using FACS sorting and magnetic cell sorting. Methods for whole cell panning have been described previously (Siegel, D.L., Chang, T.Y., Russell, S.L., and Bunya, V.Y. 1997. *J. Immunol. Methods* 206:73-85).

Prior to any selection strategy, initial libraries are electroporated into host cells (ER2537). Library cultures are grown to log phase and superinfected with helper phage, such as VCSM13, a commercially available helper phage (Stratagene, La Jolla, CA). Superinfection provides the remaining phage components needed for packaging plasmids into phagemid particles. Following overnight growth, phagemids in the culture supernate are precipitated with polyethylene glycol (PEG). PEG supernatants are used in panning (cell

surface, internalization and membrane), FACS sorting, and magnetic sorting to purify binding antibodies from non-binders.

In panning, antibody-phage libraries are incubated with target cells, and the non-adherent phage are removed with multiple washes. A typical panning protocol is as follows:

1. Block phage particles with PBS + 1%BSA or 10% FBS + 4% milk powder + NaN_3 (except when internalized antibodies are assayed).

2. Add target cells to blocked phages (approximately 5 X 10^6 cells).

3. Mix and rotate slowly at 4° C or 37 °C.

4. Wash cells twice with 1 ml ice cold PBS/1%BSA/NaN_3 or room temperature PBS/1%BSA/NaN_3.

5. Specific antibody-phage bound to cells can be eluted by low pH, for example with 76 mM citric acid ph 2.5 in PBS for 5 to 10 minutes at room temperature.

6. Neutralize eluted phage with 1M Tris-HCl pH 7.4.

7. After neutralization, antibody-phage can be used to infect ER2537 bacteria and amplify during overnight growth for the next round of panning.

Generally, 3-4 rounds of panning are performed on each library. Phage ELISAs using commercially available secondary antibody (sheep anti-M13 antibody-HRP) or soluble antibody ELISAs using a commercially available HA..11 antibody (Babco, Berkeley, CA) that recognizes the HA tag incorporated into each antibody from PRL4 sequences, can be performed on whole cells following each round of panning to allow estimation of the enrichment of binding antibodies over non-binders. Following the last round of panning, the antibody-phage can be picked as single colonies from agar plates, grown as monoclonal antibody-phage and screened by ELISA on whole cells for identification of specific binders. Specifically the antibody-phage are infected into Top10F' bacteria and plated for single colonies. Single colonies are picked form agar plates, grown and induced with IPTG. Soluble antibody is screened by ELISA on whole cells for identification of specific binders. In addition to live cells, screening can be done against intact, mildly fixed target cells.

Where the target cell can be defined by cell surface markers, relevant antibody-phage can be identified by FACS or magnetic sorting. In the case where $CD34^+$ cells were used as immunogen for example, and the desired antibody fragments bind to either $CD34^+CD38^-$ cells

or $CD34^+CD38^+$ cells, the phage-antibody library is added to the $CD34^+$ population of cells and incubated to allow antibody-phage binding to the cell surface. Following binding, cells are washed, pelleted and resuspended in fluorescein isothiocyanate (FITC)-conjugated CD38 antibody solution. After incubation, and washing, cell sorting is performed. FACS sorting has been used successfully to identify phage antibodies specific for subsets of blood leukocytes (DeKruif, J., Terstappen, L., Boel, E., Logtenberg, T. *Proc. Natl. Acad. Sci. U.S.A.* 92:3938-3942, 1995). Phage are eluted from the two populations $CD34^+$ $CD38^-$ cells or $CD34^+CD38^+$ cells at low pH, neutralized and used to infect bacterial cells. Phage or soluble antibody can then be prepared from individual colonies for further analysis. In the case of antibodies directed to the $CD34^+$ $CD38^-$ cells, phages interacting with structures present on all or a majority of cells in the heterogeneous population would be absorbed out by the $CD38^+$ cells, effectively resulting in the enrichment of phages specific for the $CD38^-$ cells. The considerable shear forces exerted on the cells and attached phage during fluorescence-activated cell sorting would result in selection for relatively high-affinity antibodies.

Alternatively, adsorber cells can be used to get rid of phage that bind non-specifically to cell surfaces or that bind to common antigens not specific to the stem/progenitor cell, such as receptors involved in cellular housekeeping functions. Adsorber cells for $CD34^+$ stem/progenitor cells, are the more differentiated $CD34^-$ cells, which can be doped in with the $CD34^+$ cells.

The phage-antibody library is added to a 1:5 mixture of $CD34^+$:$CD34^-$ cell population (for example, mononuclear cells, which are primarily $CD34^-$ can be added to presorted $CD34^+$ cells) and incubated to allow antibody-phage binding to the cell surface. Following binding, cells are washed, pelleted and resuspended in fluorescein isothiocyanate (FITC)-conjugated CD34 antibody solution and phycoerythrin (PE)-conjugated CD38 antibody solution. After incubation, and washing, cell sorting is performed to collect the two separate populations of cells; $CD34^+CD38^+$ and $CD34^+CD38^-$. Phage are eluted from the two populations at low pH, neutralized and used to infect bacterial cells. Phage or soluble antibody can then be prepared from individual colonies for further analysis. In this way, phages interacting with structures present on all or a majority of cells in the heterogeneous population would be adsorbed out

by the presence of $CD34^-$ mononuclear cells, effectively resulting in the enrichment of phages specific for the $CD34^+$ cells.

As an alternative to FACS sorting, one can also use magnetic sorting to identify antibodies/phage-antibodies specific for $CD34^+$ $CD38^-$ cells and $CD34^+CD38^+$ cells. Magnetic sorting may yield a higher background of non-specific antibodies, but may be less rigorous than FACS sorting.

If the target cell cannot be readily identified by cell surface markers, such as with NTera-2 cells, relevant antibody-phage can be identified using the same techniques of sorting by FACS or magnetic sorting in the presence or absence of excess adsorber cells. For selections in the presence of excess adsorber cell, cell populations of interest can be biotinylated, an excess of irrelevant adsorber cells is added, such as a fibroblast cell line, phage-antibodies to the mixture are bound, and the cells of interest with phage-antibodies attached are pulled out using a streptavadin-PE conjugate.

For any particular species of target cell type an adsorber cell is selected that is further differentiated than the target cell or of a distinct or unrelated cell type. Typically, the adsorber cell lacks specific surface molecules present on the target cell, but would have surface molecules common to many cells. A preferred adsorber cell is a fibroblast.

Phage displaying antibodies that recognize receptors can also be identified using an internalization protocol. For example, mammalian cells are washed with ice cold buffer (for example, PBS with 5% FBS), then exposed to phagemids displaying monomeric or dimeric antibodies for 15 minutes to one hour incubation on ice. Cells are then washed with ice cold buffer, put into fresh culture medium, and incubated at 37° C to permit internalization. Cell are then lysed to recover internalized phagemids. Phagemids can be recovered by direct infection of phagemid particles into bacterial cells, or alternatively, phagemids can be recovered by electroporation of bacterial cells, selecting for antibiotic resistance encoded by the phagemid.

Alternatively, phage displaying antibodies that recognize receptors can be screened by membrane panning. Proteins are isolated from cells of interest, for example a stem cell line, or NTera-2 cell cell line as well as from an unrelated cell type, for example a fibroblast cell line. Proteins are separated by electrophoresis on one or two dimensional polyacrylamide

gels. Following electrophoresis, proteins are transferred to a membrane support such as nitrocellulose or nylon. Proteins can be stained prior to transfer, alternatively, identical gels are run so that one can be stained with either silver or coomassie blue to detect protein bands or spots, while the other is used for membrane panning. Specific regions of the membrane may be chosen for panning based on comparisons of the cell line of interest with an unrelated cell type, alternatively larger areas of membrane containing many spots may be chosen. Patterns can be compared and regions chosen that are dissimilar between the two cell lines. These regions are cut out of the membrane as a solid support for panning. Phage are applied to the regions and allowed to bind. The membrane is washed and the phage are eluted from the membrane and infected into bacterial cells and amplified.

From the above screening methods, one can build a diverse collection of antibody binding sites displayed on the surface of phage that bind non-covalently to random cell surface targets on hematopoietic cells or other cells. After FACS sorting, magnetic sorting, or panning, clones can be analyzed individually.

Dimerization

Following panning to isolate high affinity antibody binders, bioassays for functional screens of agonist antibodies are carried out. Dimerization is often a prerequisite for activation of many receptors and thus bioassays focus on agonist antibodies that stimulate receptors via promotion of dimerization. As previously described, single chain multivalency is approached in linker design. Fab fragment multivalency can be approached in a number of ways. A number of recent reports in the literature have shown success in dimeric antibody fragment formation which is applicable to phage display (DeKruif, J., and Logtenberg, T. 1996. *J. Biol. Chem.* 271:7630-7634, Pack, P., and Pluckthun, A. 1992. *Biochemistry* 31:1579-1584, and Holliger, P., and Winter, G. 1993. *Current Opin. Biotech.* 4:446-449). Divalent Fabs can be created in at least two ways. In one approach dimerization is achieved by addition of a dimerization domain to pRL4, forming pRL8 (see Fig. 5B). There are a number of dimerization domains (lexA, Zn fingers, fos, jun etc.) that can be utilized in these vectors to obtain multivalency of Fab fragments. Dimerization domains are selected from, but not limited to, the following: jun (DeKruif, J. and Logtenberg, T. *J. Biol. Chem.* 271:7630-7634, 1996; Kostelny, S.A., Cole, M.S., and Tso, J.Y. *J.Immunol.* 148:1547-1553, 1992) the

LexA dimerization region (Kim, B. and Little, J.W. *Science* 255:203-206, 1992), the yeast GCN4 dimerization domain (van Heeckeren, W.J., Sellers, J.W., Struhl, K. *Nucleic Acids Res.* 20:3721-3724,1992), Gin invertase from the bacteriophage Mu (Spaeny-Dekking, L., Schlicher, E., Franken, K., van de Putte, P., Goosen, N. *J. Bacteriol.* 34:1779-1786, 1995), *E. coli* NTRC protein dimerization domain (Klose, K.E., North, A.K., Stedman, K.M., Kustu, S. *J. Mol. Biol.* 241:233-245, 1994), and HSV-1 ICP4 dimerization domain (Gallinari, P., Wiebauer, K., Nardi, M.C., Jiricny, J. *J. Virol.* 68:3809-3820, 1994). Also, a high temperature dimer domain from thermus organisms can be utilized (MacBeath, G., Kast, P., Hilvert, D., *Biochemistry* 37:100062-73, 1998 and MacBeath, G., Kast, P., Hilvert, D., *Science* 279:1958-61, 1998). These are functional domains that when incorporated into a molecule allow for dimerization to occur. Those of ordinary skill in the art are familiar with these and other dimerization domains and their use to dimerize proteins. Following the panning or sorting steps of Fab libraries, the library of panned molecules are restricted with *Sac* I and *Spe* I and cloned into pRL8. Subcloning to pRL8 vector individually or *en masse* following FACS sorting or panning allows expression of dimeric soluble binding Fabs for analysis in bioassays. In pRL8, the antibody fragments are transported to the periplasmic space and form dimers there. The advantage of this approach is that it permits panning of monomeric Fab fragments, favoring high affinity Fabs.

Another approach uses a secondary antibody. pRL4 has the hemagglutinin decapeptide tag recognized by the commercially available HA.11 antibody (Babco, Berkeley, CA). Fabs identified in FACS sorting or panning to be tested in bioassay are preincubated with HA.11 which will promote dimerization, prior to addition to bioassays.

Bioassays

Once binding scFv's are identified by panning, the individual clones, each expressing a unique dimerized scFv on the phage surface, are tested for proliferation, differentiation, activation or survival effects on target cells. In addition, soluble dimerized scFv's are examined in bioassays. A simple transformation of the selected phage into Top10F' will allow bacterially produced soluble scFv's to be secreted into the periplasm. Lysates of individual *E. coli* transformants can be tested for agonist effects. Fab antibodies are transferred to pRL8

(see Fig. 5B) for dimerization and expressed as soluble dimerized Fab fragments in bacterial host Top10F'.

A number of bioassays can be used in high-throughput screening. Those of ordinary skill in the art are familiar with these and other suitable bioassays. Several non-radioactive assays have been developed in which either DNA synthesis or enzyme activity can be analyzed. For example, an MTT cell proliferation assay(catalogue number G4000) (Promega Corporation, Madison, WI) that is based on an assay described by Mosmann (Mossman, T. 1983. *J. Immunol. Methods* 65:55-57) can be used. This protocol is fast and easy, and yields results within a single day. In the assay, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), a tetrazolium salt, is converted into a blue formazan product by mitochondrial dehydrogenase activity in living cells. The dehydrogenase content, and therefore the amount of colored product produced, is proportional to cell number. The colored product is detectable in an ELISA plate reader at 570nm. Assays are performed in triplicate, *en masse* in 96 well microtiter plates. Briefly, primary hematopoietic cells or growth factor dependent cell lines are plated in 100 µl aliquots in culture medium in 96-well plates. Following addition of various concentrations of antibodies or control growth factors, cells are incubated for 48-72 hours at 37°C and 5% CO_2 in a fully humidified atmosphere. MTT is added to each well, and proliferation monitored via ELISA plate reader.

For example, in proliferation assays using TF-1 cells, bacterial cells containing phagmids expressing antibodies are grown overnight at 37°C in 96 well deep well plates in 1 ml of a media that is a mixture of mammalian cell media and bacterial media (in the case of TF-1 cells: RPMI 2.7/SD 0.3/Carb 100ug/ml). TF-1 cells are a human bone marrow erythroleukemia cell line that responds to multiple cytokines (Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y.F., Miyazono, K., Urabe, A., Takaku, F., *Cell Physiol.* 140:323-334, 1989; Kitamura, T., Tojo, A., Kuwaki, T., Chiba, S., Miyazono, K., Urabe, A., Takaku, F., *Blood* 73:375-380, 1989; Kitamura, T., Takaku, F., Miyajima, A., *Int. Immunol.* 3:571-577, 1991) On the following day, the overnight cultures are subcultured 1/10 to fresh trays, and placed at 37°C for 2 hours. Following induction with IPTG at 37°C for 4 hours, the plates are centrifuged at 2000 rpm/15' at room temperature. 50 ul each culture supernate are filtered in 96 well filter trays (Millipore) to sterile 96 well assay

plates. Mammalian cells are prewashed to remove growth factor and resuspended at a concentration of 1 X 10^5 cells/ml. 50 ul cells are added to each well. Assay plates are incubated in 37°C/5% CO_2 incubator for 72 hours. At 72 hours, the trays are developed by adding 40 ul media/MTS/PMS per well. MTS is an improved more soluble version of MTT. Both assays are based on the cellular conversion of tetrazolium salt. A MTS proliferation assay kit (catalogue number G5421) can be purchased from Promega, Inc. (Madison, WI). Plates are kept at 37°C/CO_2 incubator and read at OD_{490} at 1 hour, 4 hours, 8 hours with microplate reader.

The activities of cytokines are often synergistic. Synergy could be manifested through the binding of ligands to two different receptors which then sends the correct signal, or via a priming effect whereby interaction of ligand/receptor primes the cell to respond to a second cytokine. Furthermore, cytokines that act early in lineage development are more often synergistic than cytokines that act at later stages in a developmental pathway. Therefore, suboptimal concentrations of growth factors can be used in these bioassays to examine synergism. Conditions for suboptimal concentrations are determined for each assay. This is done by adding serial dilutions of growth factors, individually and as a mixture, to the assays and determining the levels below which a single factor does not promote a response compared to the mixture, and the level below which the mixture does not promote a response in the bioassay. Bone marrow stromal cells can also be added in bioassays to provide other necessary factors that may play a role in a synergistic response.

In addition, cell proliferation can be examined by monitoring DNA synthesis. A non-radioactive, colorimetric assay that examines 5-bromo-2'-deoxy-uridine (BrdU) incorporation (Boehringer Mannheim, Indianapolis, IN) can be performed in microtiter plate format. Here, cells are cultured in 96-well plates and incubated with BrdU and sub-optimal concentrations of cytokines. The amount of BrdU is determined after labeling with a peroxidase labeled anti-BrdU antibody. Final results are analyzed by ELISA plate reader at 405nm.

A radioactive mitogenesis assay that measures the rate of DNA synthesis as an indication of proliferation (Raines and Ross, *Methods of Enzymol.* 109: 749-773, 1985) can also be used. In these assays, changes in rate of incorporation of [^{3}H]-thymidine in target cells is examined. Again, these assays permit concurrent and rapid screening of many antibody

fragments. They have been widely used as a convenient method of assessing the stimulatory and inhibitory effects on the growth of many different cells. Cells are cultured in suspension until they reach exponential growth rate. Cells are then washed free of the medium in which they were cultured, and replated in fresh medium. Cells are aliquoted into 96 well plates in a total volume of 100 ul at a concentration of about 1-2 x 10^5 cells/ml. Dilutions of phage supernatant, soluble dimerized Fab or ScFv antibodies are added and cells are incubated for 18-48 hours in a gassed CO_2 incubator at a temp of 37°C. Following incubation, [^{3}H]thymidine (937kBq) is added to each well and incubated for a further 4 hours. The cells are then removed from the incubator and counted directly in a bench top microplate scintillation counter such as Packard Top Count NXT Instrument (Packard, Meriden, CT). Alternatively cells can be serially transferred to GF/C filters on a Millipore cell harvester (Millipore, Bedford, MA) or similar apparatus. Radioactivity associated with acid-insoluble material retained on the filter is then determined. Dilutions of commercially available growth factors are applied to positive control wells. Negative controls would include supernatants from cells carrying non-insert containing plasmids or irrelevant antibodies treated similarly. The relative growth promoting activities of the standard and the diluents of the phage supernatants under test are compared to quantify the growth promoting activity in the sample.

Activation can be tested for by assaying second messengers or by transcriptional readout assays.

Survival can be assayed, for example, by monitoring apoptosis using assays such as tunel assays or by other methods known to those who practice the art.

Other useful assays to analyze cellular signal transduction, the activity of kinases and phosphatases and ultimately cellular activities as a result of agonist activity include measurement of the generation of second messengers, e.g. cAMP, Ca++, diacylglycerol (DAG), and isositol 1,4,5-triphosphate (IP3). Measurement of spikes in intracellular calcium concentration, intracellular pH and membrane potential in high throughput screening assays can be performed using instruments such as the FLIPR Fluormetric Imaging Plate Reader System (Molecular Devices, Sunnyvale, CA). A number of fluorescent probes are available for examination of second messenger concentrations (Molecular Probes, Eugene OR). Measurement of concentrations of second messengers can also be done on the single cell level

(DeBernardi, M.A. and Brooker, G. *Proc. Natl. Acad. Sci USA* 93:4577-4582, 1996). In addition, assays that examine other signaling events such as phosphorylation, apoptosis or levels of RNA or protein of specific genes would be useful. For example. most cytokines have been shown to activate the enzyme PI 3-K (reviewed in Silvennoinen, O., Ihle, J.N. Signaling by the Hematopoietic Cytokine Receptors, R.G. Landes company, Austin, TX 1996). Furthermore, the Jak family of tyrosine kinases have been shown to be central mediators for cytokine receptor signaling (Ihle, J.N., Witthuhn, B.A., Quelle, *F.W. Annu. Rev. Immunol.* 13:369-398, 1995). In addition, several other tyrosine kinases, e.g., members of the Src family, are activated in response to certain cytokine stimulations. In the case of RNA or proteins, c-Jun, c-Fos and Nfκβ are rapidly and transiently upregulated upon cytokine stimulation, while c-Myc induction is slower. These proteins are required for G1 transition and proliferation (reviewed in Silvennoinen, O., Ihle, J.N. Signaling by Hematopoietic Cytokine Receptors, R.G. Landes Company, Austin, TX 1996). High throughput screens that detect increases in these transcripts could be utilized.

In transcriptional read out assays, changes in the transcription of specific genes are observed following exposure of cells to a growth factor or growth factor mimetic (agonist or inhibitory antibody). For example, in a myc read-out assay, cells such as IL-3 dependent FDCP-mix cell line is starved of IL-3 growth factor for 8 hours, followed by exposure to growth factor mimetics, or native growth factors for 2 hours at 37°C. At this time, the cells are harvested, RNA is isolated, and reverse transcriptase-polymerase chain reactions (RT-PCR) are performed with primers specific for the myc gene. The RT-PCR reactions are electrophoresed in horizontal agarose gels for quantitation of PCR product. In this case expression of a single gene is being monitored.

Alternatively assay for changes in expression of genes can be monitored using CHIP technology, agonist antibodies could be identified under conditions of high probe sensitivity and a dynamic range. In this way, up to 10,000 could be analyzed for changes in expression. Desired genes that could be monitored could include c-myc, c-jun, NF-κβ, among others. These genes are downstream of various signal transduction pathways and their expression should change upon a mitogenic response. In one type of commercially available CHIP (Affymetrix, Santa Clara, CA), oligonucleotides from desired test genes can be printed out

onto glass surface. Target cells are exposed to test agonist antibodies. RNA is isolated from the cells exposed to test agonist antibodies, copied to cDNA, and *in vitro* transcribed in the presence of biotin. Hybridization of *in vitro* transcribed, biotinylated mRNA is used as probe in the arrays. Chips are then scanned to determine genes that show increases in transcription upon exposure to test agonist antibodies. In another version of CHIP technology (Incyte, Palo Alto, CA), the amount of DNA is not normalized on the glass, therefore, one would set up a competitive hybridization. RNA is isolated from the cells before and after exposure to agonist. cDNA is made from each sample whereby one cDNA reaction has one label incorporated, for example, Cy-3, and the other cDNA population has a different label incorporated, for example Cy-5. Signals are detected and compared on a dual laser scan to collect images.

Visual assay

All scFvs or Fabs that show a proliferative response in the above assays are also tested in traditional methylcellulose colony forming assays (Stem Cell Technologies, Vancover BC, Canada). In these assays, colony growth, and morphological changes are scored via light microscope.

Visual examination for proliferation or differentiation effects in semi-solid agar cultures or methylcellulose can be performed using unsorted or sorted primary hematopoietic cells, and stem cell lines (Stem Cell Technologies, Vancouver BC, Canada) (Eaves, C.J., Assays of hemopoietic progenitor cells. Williams Hematology 5 (eds. E. Beutler, M.A. Lichtman, B.S. Coller & T.J. Kipps), McGraw-Hill, Inc., pp L22-L26, 1995).

Hematopoietic colony assays whereby a culture medium that maximizes the growth and differentiation of hematopoietic cells is utilized. Addition of methlycellulose allows clonal progeny of a single progenitor cell to stay together and facilitates the recognition and enumeration of distinct colonies. All necessary components are added to a basic methylcellulose medium (such as Iscove's MDM, BSA, β-mercaptoethanol, L-glutamine) except colony-stimulating factor supplements. Test antibodies (phage supernatants, soluble antibodies) are added to see if they can substitute for growth factors. Hematopoietic cells in methylcellulose culture are incubated for 10-12 days following the addition of antibodies in a 37°C humidified atmosphere of 5% CO_2 in air. After 10-12 days of incubation, colonies are

counted using an inverted microscope. After another 8-10 days, colonies are counted again. Comparisons are made between media containing antibodies and controls with and without growth factors. In addition, colonies can be picked from methylcellulose and individual cells examined cytologically by staining with Wright's stain (see Atlas of Hematological Cytology, F.G.J. Hayhoe and R.J. Flemans, Wiley-InterScience 1970).

Another example of a visual assay is Pichia co-cultivation with bone marrow cells. For example, after one or more rounds of panning CD34+ or human fetal liver libraries by either FACS, or magnetic selection, the antibody genes are cloned from the panned library into a Pichia expression vector so the antibody genes are under control of the pGAP promoter and secreted from cells using the α-factor leader sequence (InVitrogen, Carlsbad, CA) (Das, R.C., Shultz, J.L., Leluman, D.J., *Mol. Gen. Genet.* 218:240-8, 1989). Pichi atransformants are selected on YPD + Zeocin. Colonies are replica plated to "Iscoves plates" (Iscoves media plus agar). Cells are scraped off plates in 5 ml Iscoves media, centrifuged, resuspended in 0.6 ml Iscoves media and counted. Methylcellulose medium that does not contain added growth factors (catalogue #4230 Stem Cell Technologies, Vancouver BC, Canada) is thawed. 5000 Pichia cells and 50,000 human bone marrow CD34+ cells are plated in the medium per 3 cm dish. Plates are incubated in a humidified atmosphere at 37°C 5% CO2. Scoring is done at 5-12 days. If a bone marrow colony is noted, the nearby yeast is plucked from the methylcellulose, and retested in a similar way. The antibody genes can be fingerprinted from Pichia by a whole cell PCR and restriction digest with EcoRII. The antibody released into the medium can be purified and used in FACS sorting to examine the population of cells the antibody binds to, in the presence of other known fluorescein conjugated antibodies. Here, the primary antibody is secreted from yeast. The secondary antibody is HA.11-FITC conjugate (Babco, Berkeley, CA).

Stem Cell Assays

Candidates identified in stem cell specific assays are further analyzed for their ability to stimulate renewal in serum free medium (Stem Cell Technologies, Vancouver BC, Canada), and the ability of cells cultured in the presence of agonists to generate long term culture initiating cells (LTC-IC) (Stem Cell Technologies, Vancover BC, Canada). Agonists that appear to expand LTC-IC *in vitro* are subjected to DNA analysis, including restriction

enzyme footprinting and DNA sequencing, in order to identify unique clones. Unique agonists are further assayed to determine whether cells cultured in the presence of the agonist support long term engraftment in irradiated mice (Hodgson, G.S. and Bradley, T.R. *Nature* 281:381-382, 1979; Harrison, D.E. *Blood* 55:77-81, 1980) or in NOD/SCID mice (Conneally, E., Cashman, J., Petzer, A., Eares, C. *Proc. Natl. Acad. Sci. U.S.A.* 94: 9836-9841, 1997). Agonists that prove interesting by these criteria are subject to sequence analysis, binding studies, and used to identify the receptor through immunoprecipitation, protein sequence analysis, and database searches.

Receptor effects

Even if the receptor is unknown, if the receptor encodes a kinase or the receptor causes kinase activity, receptor phosphorylation can be examined by incubating receptor-containing membrane extracts with [γ-32P]ATP in the presence of the test compound (antibody). Then the extracts are examined for receptor phosphorylation by gel electrophoresis and autoradiography. These and other techniques for examining receptor phosphorylation are known to those who practice the art.

Synthesis of antibodies

Once antibody fragments are identified in bioassays they are selected for high level expression as soluble antibody fragments. Soluble ScFv fragments or Fab fragments can be isolated in bacterial hosts such as Top10F' (Invitrogen, Carlsbad, CA).

Single colonies of cells are grown up and the soluble antibody fragments are purified by methods such as nickel-chelate chromatography, using the His6 sequence engineered in the vectors.

Those of ordinary skill in the art using known techniques would be able to synthesize antibodies in other organisms such as yeast, mammalian, insect, and plants (Carlson, J.R. and Weissman, I.L., *Mol. Cell. Biol.*, 8:2647-2650, 1988; Trill, J.J., Shatzman, A.R., Ganguly, S. *Curr. Opin. Biotechnol.* 6:553-560, 1995; Hiatt, A., Cafferkey, R. Bowdish, K. *Nature* 342: 76-78, 1989).

Example 2: Identification of the growth factor receptor

The first generation agonist molecules are grouped into categories depending on cell type that appears to be proliferating and/or differentiating. The receptor can be identified by using a number of approaches including immunoprecipitation and affinity chromatography and chemical crosslinking followed by protein sequencing and database searches.

In immunoprecipitation, cells can be radiolabelled, lysed in the presence of detergent such as Triton X-100, and the binding proteins precipitated by the antibody, and analyzed by SDS-PAGE followed by autoradiography. Therefore this will allow detection of the receptor, characterization of its molecular size, and identification of other subunits or associated proteins. Additionally, proteins can be electrophoretically transferred to a sheet of polyvinylidene difluoride (PVDF) and stained with Comassie blue. The separated bands can be excised, and analyzed in an automated protein sequencer, followed by data base searches to determine whether the receptor is new, or previously identified. Additionally, the proteins can be cleaved with proteolytic enzymes to determine internal amino acid sequences.

In conventional immunoaffinity purification, the antibody is covalently attached to a solid-phase matrix. Typically, antibodies are coupled to Sepharose. A cell lysate, prepared in the presence of detergent such as Triton X-100 for lysis and solubilization of integral membrane proteins is prepared. Lysates are applied to the columns, the column is washed, and the binding proteins are eluted by brief exposure to high-pH, or low-pH buffer. Each is followed by partial amino acid sequencing, and database searches. Alternatively, specific elution can be done with soluble scFv. Often a precolumn is used to reduce non-specific binding. If multiple species are present, one can determine which is the ligand-binding subunit for the agonist by cell surface radioreceptor cross-linking of radiolabeled scFv to intact cells.

An expression cloning approach could also be used. A cDNA library is constructed from cells responding to the antibody. The library is cloned into a mammalian expression vector and transfected into mammalian cells. Cells expressing the receptor are identified by affinity chromatography or "routine panning" where a single species of antibody-phage are placed in contact with a pool of cells, each expressing a different cDNA clone.

Once the receptor is identified it can be cloned using standard molecular biology techniques. Transient transfection assays with cDNAs encoding the receptor in mammalian cells can be carried out to demonstrate that it is the receptor the antibody recognizes. For example COS cells can be utilized for receptor expression (Neil, J.D., Sellers, J.C., Musgrove, L.C., Duck, L.W. *Mol. Cell. Endrocrinol.* 127:143-154, 1997).

Identification of the natural ligand

The native ligand of a newly identified receptor can be identified by expressing the receptor heterologously in mammalian cells or in yeast, preferably in cells that normally do not express the receptor. Pools of secreted factors from expressed cDNA libraries can be added to receptor expressing cells and analyzed in the same bioassays used to identify the agonist (see Lee, F. et al. *Proc. Natl. Acad. Sci.* 82:4360, 1985). In addition to transient expression systems, permanent expression systems in retroviral vectors can also be used in these assays (Rayner, J.R. and Gonda, T.J. *Mol. Cell. Biol.*, 14:880, 1994). A positive response is linked back to an individual clone expressing the specific ligand cDNA. In addition, competition experiments can be performed with the ligand and the agonist antibody where one is labeled with ^{125}I or fluorescent compounds.

Those of ordinary skill in the art are familiar with these techniques and others useful for identifying the native ligand once the receptor is known.

Screening for inhibitory antibodies

Inhibitory antibodies are screened using the same assays as utilized to screen for agonist antibodies, panning and bioassays. In bioassays however, additional growth factors, such as IL-3, are included in the culture to enable the cells to grow, and inhibition of proliferation, differentiation, survival or activation is observed.

Example 3: Phage display libraries to hematopoeitic cells and other cell types

Animals (rabbits) were immunized individually with the following cells or cell lines: human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, human bone marrow cells that have undergone RBC lysis, human fetal liver cells, human NTera-2 cell line, murine P19 cell line, and mouse FDCP-mix cell line. Rabbits were immunized with whole

cells, according to the protocol in Example 1, 3-5 times in 3 week intervals until a strong positive response was detected.

Prior to immunization, approximately 5 ml of blood was obtained from each animal. The blood was processed to serum immediately by allowing the blood to clot, centrifuging and pelleting of cells, and transferring serum to fresh tubes. Serum from each animal was stored at –20°C. After primary immunizations and two boosts, blood was again collected from each animal and processed to serum. Whole cell ELISAs were performed comparing preimmune serum to post-immune serum. Dilutions of serum were made, e.g., 1/50, 1/100, 1/500, 1/1000, 1/5000 in 5% milk in PBS. Cells of the same type used for the immunization(1×10^6) were applied to plates, serum dilutions were added and the cells were incubated. The secondary antibody was an enzyme-conjugated antibody to the host animal IgG that is being tested, for example goat anti-rabbit IgG-alkaline phosphatase conjugate or peroxidase conjugate. Results were read visually or plates were centrifuged, supernatants removed and placed in fresh wells for ELISA plate reading. Readings are graphed to determine the half-maximum serum response, or that dilution that gives a response that is equal to one half of the maximum response.

When a positive response was detected, the animals were sacrificed and spleen, bone marrow and peripheral blood lymphocytes were collected. RNA was isolated from these organs and libraries were constructed as in Example 1 to produce antibody fragment libraries displayed on the surface of phagemids. Ligations were electroporated into ER2537. A small aliquot was plated to determine efficiency of cloning and potential diversity of libraries as in Example 1. Results are shown in Table 2.

Table 2

Name/Type of Ab	Antigen	Serum Response	Potential Diversity
B1/scFv short	hBM mononuclear cells	1/300	5.2×10^7
B2/scFv long	hBM mononuclear	1/300	1.3×10^8
B3/chimeric Fab	hBM mononuclear	1/300	1.6×10^8
F1/scFv short	hBM CD34+	1/400	3.0×10^8
F2/scFv long	hBM CD34+	1/400	1.5×10^8
F3/chimeric Fab	hBM CD34+	1/400	5.2×10^8

Name/Type of Ab	Antigen	Serum Response	Potential Diversity
G1/scFv short	hBM CD34+	1/1000	2.3×10^8
G2/scFv long	hBM CD34+	1/1000	1.9×10^8
J1/scFv short	hBM RBC lysis	1/250	3.5×10^8
J2/scFv long	hBM RBC lysis	1/250	4.1×10^8
K1/scFv short	hBM RBC lysis	1/1000	5.3×10^8
K2/scFv long	hBM RBC lysis	1/1000	4.9×10^8
T1/scFv short	h fetal liver	1/1000	1.8×10^9
T2/scFv long	h fetal liver	1/1000	1.9×10^9
U1/scFv short	h fetal liver	1/750	5×10^8
U2/scFv long	h fetal liver	1/750	1.6×10^9
PQ1/scFv short	n NTera-2	1/500	nd
PQ2/scFv long	h NTera-2	1/500	nd
MN1/scFv short	m P19	1/500	1.0×10^9
MN2/scFv long	m P19	1/500	2.5×10^9
BBCC1/ scFv short	m FDCP-mix	1/1000	nd
BBCC2/ scFv long	m FDCP-mix	1/1000	nd

h = human
BM = bone marrow
m = murine
nd = actual value not determined yet.

Those of ordinary skill in the art will recognize that using the above described protocols other animals, such as chickens, and could be immunized with other cell types and lines such as yolk sac cells, cells derived from the murine AGM region, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, in order to generate additional libraries of phage displayed antibody fragments to a variety of receptors on a number of cell types.

Pharmaceutical Formulations And Routes Of Administration

The agents described herein can be administered to a human patient *per se*, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s). Techniques for formulation and administration of the compounds of the instant application

may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

Routes Of Administration

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the agents in a local rather than systemic manner.

Furthermore, one may administer the agents in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

Composition/Formulation

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, *e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active molecules into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the agents can be formulated readily by combining the active molecules with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be

treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the agents may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the agents for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to

deliver a metered amount. Capsules and cartridges of *e.g.* gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The agents may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active molecules in water-soluble form. Additionally, suspensions of the active molecules may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The agents may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the agents may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the agents may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic agents of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, *e.g.* polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the agents of the invention may be provided as salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids,

including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.

Effective Dosage

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount of agent effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any agent used in the methods of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC_{50} as determined in cell culture (*e.g.*, the concentration of the test molecule which promotes or inhibits cellular proliferation or differentiation). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the agents described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD_{50} and ED_{50}. Molecules which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such molecules lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual

physician in view of the patient's condition. (See *e.g.*, Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to promote or inhibit cellular proliferation or differentiation or minimal effective concentration (MEC). The MEC will vary for each agent, but can be estimated from *in vitro* data using described assays. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

In cases of local administration or selective uptake, the effective local concentration of the agent may not be related to plasma concentration.

The amount of agent administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

Therapy

Antibodies and factors encompassed by the claimed invention are useful for the amplification of a variety of clinically relevant cell types. Treatment can be *in vivo* or *ex vivo*. For example, agonist antibodies are useful to treat patients suffering from a deficiency in a hematopoietic cell population caused by disease, disorder or treatment related to for example suppression of hematopoiesis where less than the normal number of cells of a given lineage or lineages are present in a patient. The following represent only some examples of the conditions that can be treated with the antibodies and factors of the claimed invention, those who practice the art would be able to identify other diseases and conditions that would benefit from such treatment. For example, HIV-infected patients, patients undergoing chemotherapy, bone marrow transplant patients, and patients suffering from myeloproliferative disorders show subnormal levels of specific hematopoietic lineages. Neutropenia is a decrease in the number of circulating, terminally differentiated neutrophils. Neutropenia appears secondary to or in association with a number of pathophysiologic conditions, including viral and severe

bacterial infections; exposure to drugs, such as certain antibiotics and anticonvulsants; autoimmune processes, such as those seen with systemic lupus erythematosus; marrow infiltrative processes, such as leukemia, metastatic tumor, and myelofibrosis; and inborn errors of metabolism, such as propionic acidemia and isovaleric acidemia. Some chronic primary neutropenic disorders such as cyclic neutropenia are transmitted genetically, while others occur in association with disorders of immune function, such as that produced by infection with Epstein-Barr or human immunodeficiency virus. Other conditions may be associated with chronic idiopathic neutropenia including Shwachman syndrome, X-linked agammaglobulinemia, dysgammaglobulinemia, and depressed cellular immunity. Acquired agranulocytosis is a blood disorder characterized by a reduction in the number of granular leukocytes in the circulating blood due to an impairment in granulocyte production in bone marrow. Patients undergoing renal dialysis often suffer from treatment related anemia with subnormal levels of red blood cells. In aplastic anemia, bone marrow suppression can cause pancytopenia or may affect only the red blood cells, the white cells, or the platelets. Agonist antibodies and factors will augment the armamentarium of therapeutic agents for these and other diseases and disorders characterized by deficiencies in specific cell populations, such as hematopoietic cells.

Antibodies and factors encompassed by the claimed invention are also useful for the inhibition of clinically relevant cell types. For example, abnormal growth of hematopoietic cell populations include diseases where greater than the normal number of cells of a given lineage or lineages are present in a patient. Chronic myeloid leukemia is a disease characterized by myeloid proliferation due the presence of a chromosomal translocation in the hematopoietic stem cells. Thrombocytopenia is a disease characterized by an increase in the numbers of megakaryocytes. Polycythemia vera is a form of myeloproliferative syndrome in which the granulocyte, monocyte, and platelet counts as well as the red cell count are usually elevated. This disorder is caused by a neoplastic clone of stem cells which proliferate excessively. In allergic diseases such as rhinitis, asthma, and exzema, there is excessive production of basophils, mast cells and eosinophils. Inhibitors of eosinophil, mast cell and basophil production identified by the claimed invention can serve as therapeutics for severe allergy sufferers. In addition, inhibitors can be used clinically in chemotherapy to inhibit the

proliferation of normal cells, while cancerous cells will continue through the cell cycle and therefore be more susceptible to chemotherapeutic agents.

The molecules encompassed by the claimed invention can also be used for *ex vivo* proliferation and differentiation of cells. This is useful for gene therapy purposes, for example for traditional viral vector approaches, and for autologous bone marrow transplants. Gene transfer into hematopoietic progenitor cells is a major goal of gene therapy for malignant and non-malignant disease. Most gene therapy vectors in use require mitosis for integration of viral genome into cellular DNA. Integration is preferred since a single stem cell will divide to form many progeny cells. Agonist antibodies, factors, or combinations of the above administered before or during vector exposure can promote proliferation of stem cells *ex vivo*.

In addition, antibodies of the present invention can be radiolabeled for radioimmunotherapy or conjugared to toxins to deliver such toxins to specific cell types and result in the killing of those cells.

Diagnosis and Purification

The molecules encompassed by the claims can be used in diagnostics where the antibody fragments specific to a cell lineage are conjugated to fluorescent markers or used as primary antibodies with secondary antibodies that are conjugated to fluorescent markers, and used in flow cytometry analysis to identify clinically relevant cell types, such as in the blood or bone marrow of a patient. In addition, the molecules can be used in cell isolation strategies such as fluorescence-activated cell sorting (FACS), or magnetic sorting procedures. In fluorescence-activated cell sorting, cells tagged with fluorescent molecules are sorted electronically on a flow cytometer such as a Becton-Dickinson (San Jose, California) FACS IV cytometer or equivalent instrument. The fluorescent molecules are antibodies that recognize specific cell surface antigens. The antibodies are conjugated to fluorescent markers such as fluorescein isothiocyanate (FITC) or Phycoerythrin (PE). In magnetic sorting procedures, the antibody is linked directly or indirectly to magnetic microbeads. Cells are precoated with agonist or inhibitory antibodies that recognize cell surface molecules, e.g., receptors involved in proliferation, differentiation, activation or survival. The antibodies are attached to magnetic beads conjugated with a secondary immunoglobulin that binds to the agonist or inhibitory antibody, such as to the HA molecular tag engineered into each antibody.

The cells are then removed with a magnet. Magnetic sorting can be positive selection where cells of interest are bound by the antibody and hence the magnet, or negative selection where undesired cells are isolated onto the magnet.

Alternatively, radiolabeled antibodies can be used for diagnostic purposes.

Other embodiments are within the claims.

CLAIMS

1. Method to identify agonist or inhibitory antibodies to receptors involved in cellular proliferation, differentiation, survival or activation comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising said receptors, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said antibodies are surface displayed, and

screening said surface displayed antibodies using target cells to identify agonist or inhibitory antibodies to said receptors.

2. Method to identify agonist antibodies to growth factor receptors comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising said growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said antibodies are surface displayed, and

screening said surface displayed antibodies using target cells to identify agonist antibodies to said growth factor receptors.

3. The method of claim 2, wherein said antibodies are scFv fragments.

4. The method of claim 2, wherein said antibodies are Fab fragments.

5. Method of claim 3, wherein said surface display vectors are phagemid vectors.

6. Method to identify agonist antibodies to growth factor receptors comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising said growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of said antibodies,

cloning said nucleic acid sequences from said library into phagemid vectors so that said scFv fragments are displayed on the surface of phagemid,

panning said scFv fragments displayed on phagemids for binding to cell surface molecules on target cells, and

screening in a functional assay said scFv fragments that bind said cell surface molecules to identify those that are agonist antibodies for said growth factor receptors.

7. Method to identify agonist antibodies to growth factor receptors comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising said growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said Fab fragments are surface displayed,

panning said surface displayed Fab fragments for binding to cell surface molecules on target cells,

dimerizing said Fab fragments that bind said cell surface molecules, and

screening in a functional assay said dimerized Fab fragments to identify those that are agonist antibodies for said growth factor receptors.

8. The method of claim 7, wherein said surface display vectors are phagemid vectors.

9. The method of claims 1 or 2 wherein said stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

10. Method to make agonist antibodies to growth factor receptors comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising said growth factor receptors, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said antibodies are surface displayed,

screening using target cells said surface displayed antibodies to identify antibodies that are agonists antibodies for said growth factor receptors, and

synthesizing said agonist antibodies.

11. The method of claims 1 or 2 wherein said library is a combinatorial library.

12. Method for screening for agonist antibodies comprising the steps of:

growing cells expressing antibody fragments in the presence of target cells expressing receptors to which said antibodies are directed, and

screening said antibody fragments to identify those that are agonist antibodies.

13. Agonist antibodies to growth factor receptors produced by immunizing an animal with stem/progenitor cells.

14. The antibodies of claim 13, where said stem/progenitor cell are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

15. Agonist antibodies to growth factor receptors produced by the method of claim 10.

16. Combinatorial library produced from immune cells generated by immunizing an animal with stem/progenitor cells.

17. Combinatorial library encoding antibody molecules or fragments thereof comprising nucleic acid sequences from immune cells of animals immunized with stem/progenitor cells.

18. Combinatorial antibody fragment library directed to surface molecules on stem/progenitor cells.

19. Combinatorial library produced by immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation, survival or activation, so as to generate a plurality of immune

cells expressing one or more antibodies or antibody fragments thereof to said surface molecules,

creating a library from said plurality of immune cells comprising nucleic acid sequences encoding said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said antibodies are surface displayed.

20. The library of any of claims 16, 17, 18, or 19 wherein said stem/progenitor cells are selected from the group consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

21. The library of any of claims 16, 17, 18, or 19, wherein said antibodies are scFv fragments.

22. The library of claims 16, 17, 18, or 19, wherein said antibodies are Fab fragments.

23. The library of any of claims 16, 17, 18, or 19, wherein the antibodies or fragments thereof encoding by said combinatorial library are surface displayed on phagemids.

24. Method of producing a combinatorial antibody library encoding antibodies or fragments thereof to receptors involved in cellular proliferation, differentiation, survival or activation comprising the steps of;

immunizing an animal with stem/progenitor cells having surface molecules comprising said receptors, so as to generate a plurality of immune cells expressing one or more antibodies or antibody fragments to said surface molecules,

obtaining nucleic acid sequences encoding variable and constant regions of said antibody fragments, from said plurality of immune cells,

randomly combining said nucleic acid sequences encoding variable regions of said antibody fragments to produce a combinatorial library of nucleic acid sequences encoding said antibody fragments, and

cloning said nucleic acid sequences of said library into surface display vectors so that said antibody fragments are surface displayed.

25. The method of claim 24, further comprising the step of screening serum obtained from said immunized animal for binding to said stem/progenitor cell, prior to obtaining nucleic acid sequences encoding said antibody fragments.

26. The method of claim 24, where said stem/progenitor cells are selected from the group of cells consisting of unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

27. Method to identify inhibitory antibodies for cellular proliferation differentiation or activation comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to

generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said antibodies are surface displayed, and

screening said surface displayed antibodies for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

28. The method of claim 27, wherein said antibodies are scFv fragments.

29. The method of claim 27, wherein said antibodies are Fab fragments.

30. Method of claim 28, where said surface display vectors are phagemid vectors.

31. Method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding scFv fragments of said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said scFv fragments are surface displayed,

panning said surface displayed scFv fragments for binding to cell surface molecules on target cells, and

screening in a functional assay said scFv fragments that bind said cell surface molecules for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

32. Method to identify inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding Fab fragments of said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said Fab fragments are surface displayed,

panning said surface displayed Fab fragments for binding to cell surface molecules on target cells,

dimerizing Fab fragments that bind said cell surface molecules, and

screening in a functional assay both monomer and dimerized Fab fragments for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

33. The method of claims 31 or 32, wherein said surface display vectors are phagemid vectors.

34. The method of claim 27, wherein said stem/progenitor cells are selected from the group consisting unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

35. Method to make inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation or activation, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said antibodies are surface displayed,

screening said surface displayed antibodies for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies, and

synthesizing said inhibitory antibodies.

36. The method of claim 27, wherein said library is a combinatorial library.

37. Method for screening for inhibitory antibodies for cellular proliferation, differentiation or activation comprising the steps of:

growing cells expressing antibody fragments in the presence of target cells expressing receptors to which said antibodies are directed, and

screening said antibody fragments for those that inhibit proliferation, differentiation or activation of target cells so as to identify inhibitory antibodies.

38. Inhibitory antibodies to receptors involved in cellular proliferation, differentiation or activation made by immunizing an animal with stem/progenitor cells.

39. The inhibitory antibodies of claim 38, wherein said stem/progenitor cells are selected from the group consisting unsorted human bone marrow cells, human peripheral blood cells originating from human bone marrow, sorted human bone marrow cells, unsorted murine bone marrow cells, sorted murine bone marrow cells, fetal liver cells, yolk sac cells, cells derived from the murine AGM region, human or murine embryonal carcinoma cells or

lines, human or mouse pluripotential teratocarcinoma cells or lines, murine pluripotent embryonic cells, human embryonic stem (ES) cell lines, cells of neural origin, cells involved in organ or tissue regeneration, human bone marrow cells that have undergone RBC lysis, human bone marrow mononuclear cells, human bone marrow $CD34^+$ cells, FDCP-mix murine hematopoietic stem cell line, B6SUTA murine hematopoietic stem cell line, P19 teratocarcinoma cells, and NTera-2 pluripotent embryonal carcinoma cells.

40. Inhibitory antibodies to receptors involved in cellular proliferation, differentiation or activation made by the method of claim 35.

41. Method to identify inhibitory antibodies for cellular proliferation differentiation, activation or survival comprising the steps of:

immunizing an animal with stem/progenitor cells having surface molecules comprising receptors involved in cellular proliferation, differentiation, activation or survival, so as to generate a plurality of immune cells expressing one or more antibodies to said surface molecules,

creating a library from said plurality of immune cells, comprising nucleic acid sequences encoding said antibodies,

cloning said nucleic acid sequences from said library into surface display vectors so that said antibodies are surface displayed, and

screening said surface displayed antibodies for those that inhibit proliferation, differentiation, activation or survival of target cells so as to identify inhibitory antibodies.

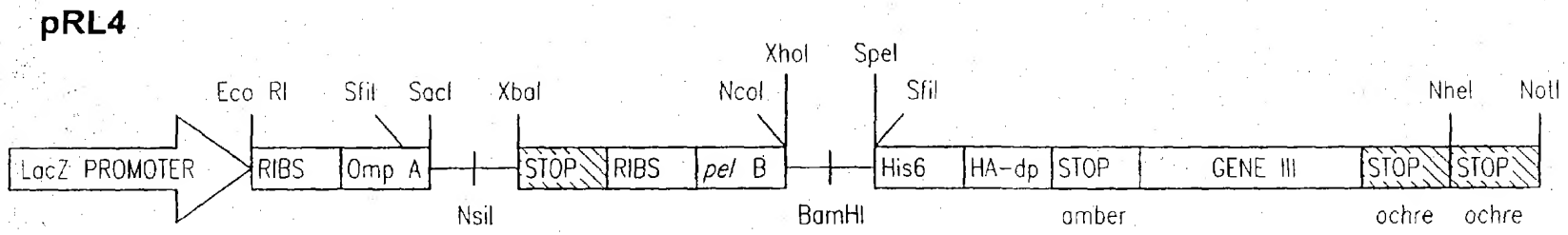


FIG. 1

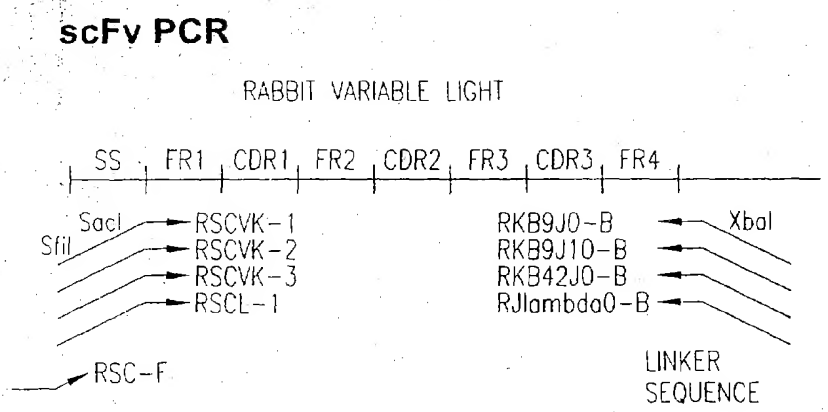


FIG. 2a

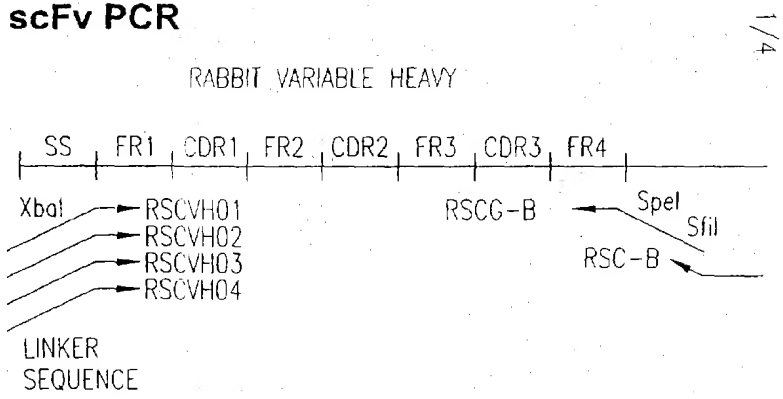


FIG. 2b

Fab PCR

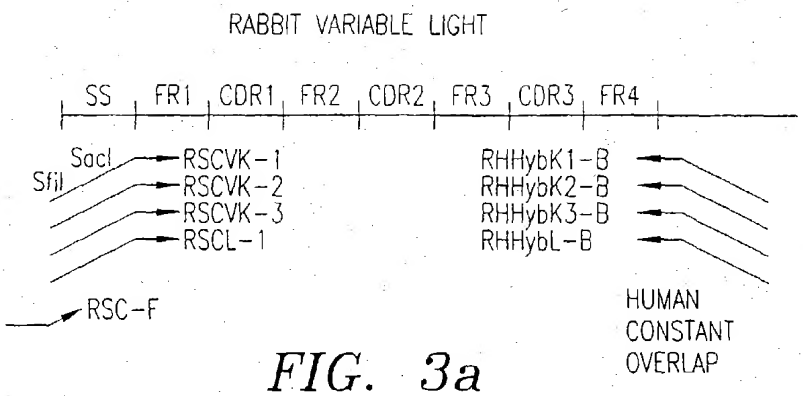


FIG. 3a

Fab PCR

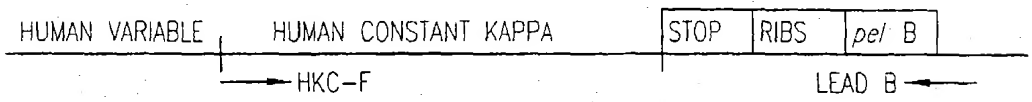


FIG. 3b

Fab PCR

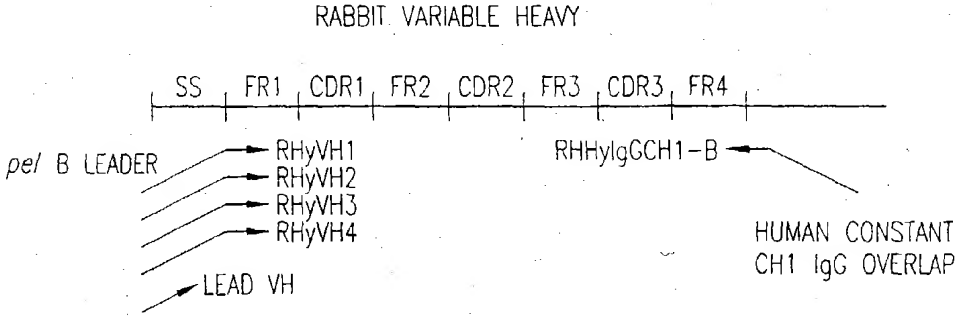


FIG. 3c

Fab PCR

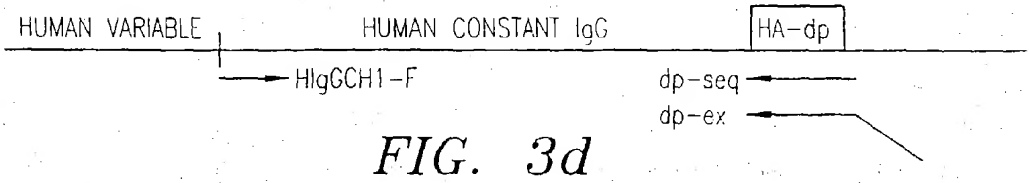


FIG. 3d

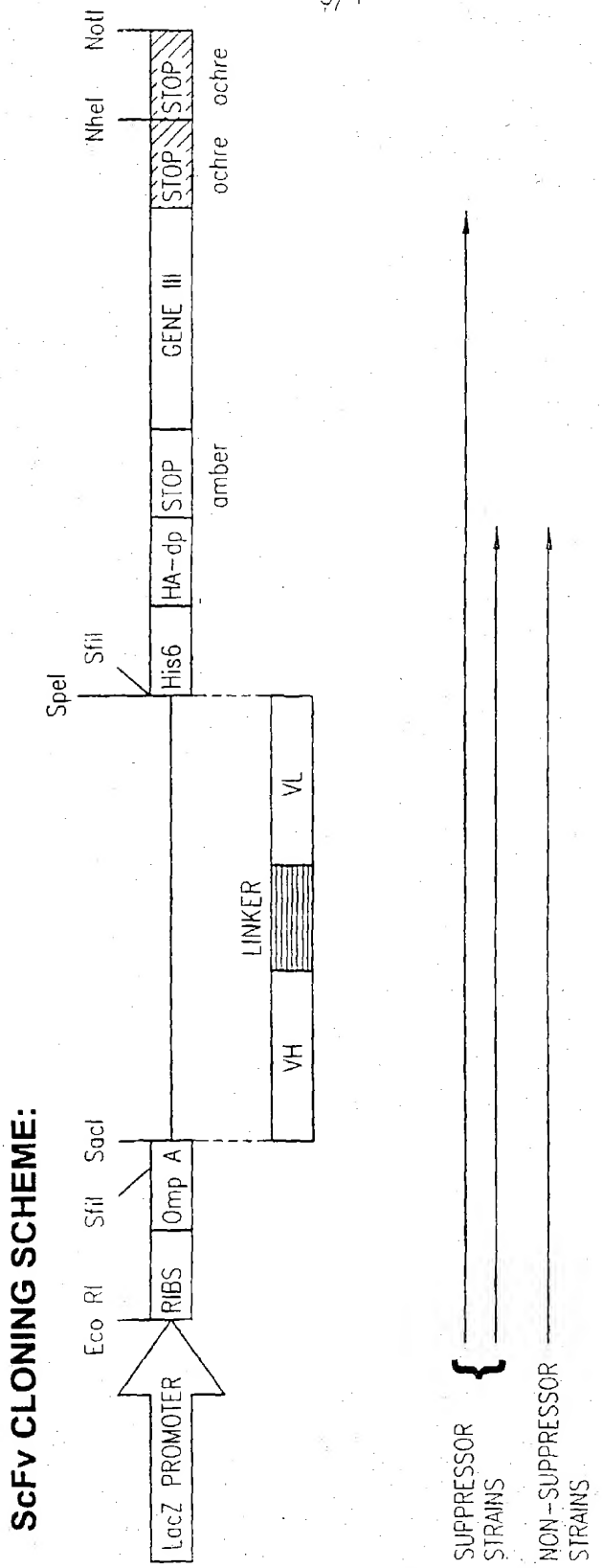

ScFv CLONING SCHEME:
LacZ PROMOTER
Eco RI
RIBS
SfiI
SacI
Omp A
VH
LINKER
VL
SpeI
SfiI
His6
HA-dp
STOP
amber
GENE III
STOP
STOP
ochre
ochre
NheI
NotI
SUPPRESSOR STRAINS
NON-SUPPRESSOR STRAINS

FIG. 4

pRL4

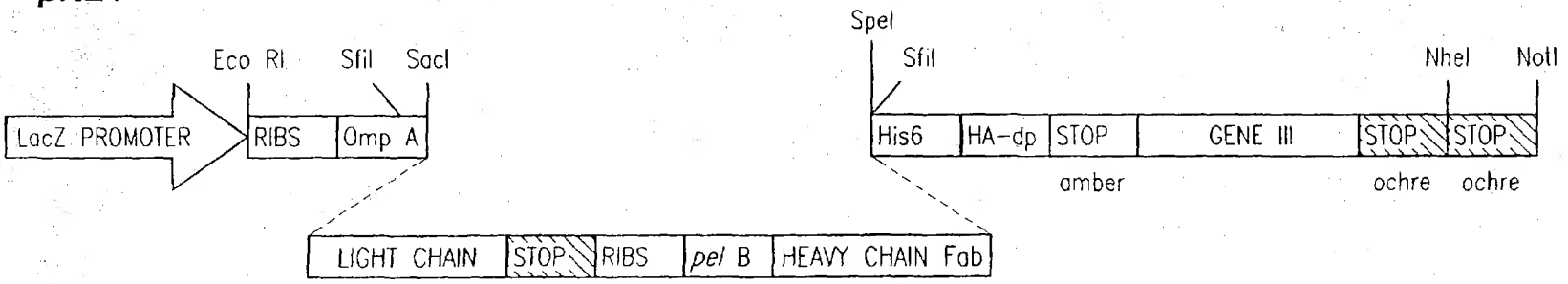


FIG. 5a

pRL8

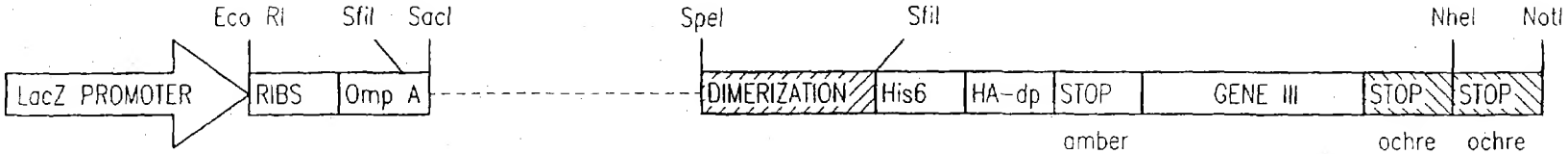


FIG. 5b

SEQUENCE LISTING

<110> PROLIFARON, LLC

<120> METHODS AND COMPOSITIONS FOR THE IDENTIFICATION OF GROWTH FACTOR MIMETICS, GROWTH FACTORS AND INHIBITORS

<130> 239/152-PCT

<140> TO BE ASSIGNED
<141> HEREWITH

<150> US 60/072,253
<151> 1998-01-23

<160> 36

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 60
<212> DNA
<213> SYNTHETIC

<400> 1

caccatggcg catacccgta cgacgttccg gactacgctt cttaggaggg tggtggctct 60

<210> 2
<211> 23
<212> DNA
<213> SYNTHETIC

<400> 2

gcttacaatt tcccagatct gcg 23

<210> 3
<211> 70
<212> DNA
<213> SYNTHETIC

<400> 3

gaggaggagg aggaggagac tagtggccag gccggccagc accatcacca tcaccatggc 60
gcatacccgt 70

<210> 4
<211> 38
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "m" stands for a or c.

<400> 4

gggcccaggc ggccgagctc gtgmtgaccc agactcca 38

<210> 5
<211> 38
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "m" stands for a or c.

<400> 5

gggcccaggc ggccgagctc gatmtgaccc agactcca 38

<210> 6
<211> 38
<212> DNA
<213> SYNTHETIC

<400> 6

gggcccaggc ggccgagctc gtgatgaccc agactgaa 38

<210> 7
<211> 40
<212> DNA
<213> SYNTHETIC

<400> 7

gggcccaggc ggccgagctc gtgctgactc agtcgccctc 40

<210> 8
<211> 42
<212> DNA
<213> SYNTHETIC

<400> 8

ggaagatcta gaggaaccac ctaggatctc cagctcggtc cc 42

<210> 9
<211> 42
<212> DNA
<213> SYNTHETIC

<400> 9

ggaagatcta gaggaaccac ctttgatttc cacattggtg cc 42

<210> 10
<211> 42
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "s" stands for c or g.

<400> 10

ggaagatcta gaggaaccac ctttgacsac cacctcggtc cc 42

<210> 11
<211> 45
<212> DNA
<213> SYNTHETIC

<400> 11

ggaagatcta gaggaaccac cgcctgtgac ggtcagctgg gtccc 45

<210> 12
<211> 42
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "r" stands for a or g.

<400> 12

ggtggttcct ctagatcttc ccagtcggtg gaggagtccr gg 42

<210> 13
<211> 42
<212> DNA
<213> SYNTHETIC

<400> 13

ggtggttcct ctagatcttc ccagtcggtg aaggagtccg ag 42

<210> 14
<211> 42
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "y" stands for c or t.

<400> 14

ggtggttcct ctagatcttc ccagtcgytg gaggagtccg gg 42

<210> 15
<211> 44
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "s" stands for c or g.
The letter "r" stands for a or g.

<400> 15

ggtggttcct ctagatcttc ccagsagcag ctgrtggagt ccgg 44

<210> 16
<211> 46
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "y" stands for c or t.

<400> 16

cctggccggc ctggccacta gtgactgayg gagccttagg ttgccc 46

<210> 17
<211> 41
<212> DNA
<213> SYNTHETIC

<400> 17

gaggaggagg aggaggaggc ggggcccagg cggccgagct c 41

<210> 18
<211> 41
<212> DNA
<213> SYNTHETIC

<400> 18

gaggaggagg aggaggagcc tggccggcct ggccactagt g 41

<210> 19
<211> 42
<212> DNA
<213> SYNTHETIC

<400> 19

agatggtgca gccacagttc gtttgatttc cacattggtg cc 42

<210> 20
<211> 42
<212> DNA
<213> SYNTHETIC

<400> 20

agatggtgca gccacagttc gtaggatctc cagctcggtc cc 42

<210> 21
<211> 42
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "s stands for c or g.

<400> 21

agatggtgca gccacagttg ctttgacsac cacctcggtc cc 42

<210> 22
<211> 45
<212> DNA
<213> SYNTHETIC

<400> 22

agatggtgca gccacagttc ggcctgtgac ggtcagctgg gtccc 45

<210> 23
<211> 24
<212> DNA
<213> SYNTHETIC

<400> 23

cgaactgtgg ctgcaccatc tgtc 24

<210> 24
<211> 21
<212> DNA
<213> SYNTHETIC

<400> 24

ggccatggct ggttgggcag c 21

<210> 25
<211> 42
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "r" stands for a or g.

<400> 25

gctgcccaac cagccatggc ccagtcggtg gaggagtccr gg 42

<210> 26
<211> 42
<212> DNA
<213> SYNTHETIC

<400> 26

gctgcccaac cagccatggc ccagtcggtg aaggagtccg ag 42

<210> 27
<211> 42
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "y" stands for c or t.

<400> 27

gctgcccaac cagccatggc ccagtcgytg gaggagtccg gg 42

<210> 28
<211> 44
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "s" stands for c or g.
The letter "r" stands for a or g.

<400> 28

gctgcccaac cagccatggc ccagsagcag ctgrtggagt ccgg 44

<210> 29
<211> 45
<212> DNA
<213> SYNTHETIC

<220>
<223> The letter "r" stands for a or g.
The letter "y" stands for c or t.

<400> 29

cgatgggccc ttggtggagg ctgargagay ggtgaccagg gtgcc 45

<210> 30
<211> 24
<212> DNA
<213> SYNTHETIC

<400> 30

gcctccacca agggcccatc ggtc 24

<210> 31
<211> 21
<212> DNA
<213> SYNTHETIC

<400> 31

agaagcgtag tccggaacgt c 21

<210> 32
<211> 21
<212> DNA
<213> SYNTHETIC

<400> 32

gctgcccaac cagccatggc c 21

<210> 33
<211> 39
<212> DNA
<213> SYNTHETIC

<400> 33

gaggaggagg aggaggagag aagcgtagtc cggaacgtc 39

<210> 34
<211> 15
<212> DNA
<213> SYNTHETIC

<400> 34

ggaagaagag gaacc 15

<210> 35
<211> 21
<212> DNA
<213> SYNTHETIC

<400> 35

ggtggttcgt ctagatcttc c 21

<210> 36
<211> 33
<212> DNA
<213> SYNTHETIC

<400> 36

cccaccaccg cccgagccac cgccaccaga gga 33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01331

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 33/53, 33/536; C12N 15/63; C12Q 1/68; C07H 21/04

US CL : 435/7.1, 320.1, 6; 436/536

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 320.1, 6; 436/536

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS

search terms: stem/progenitor cells, agonist, inhibitory antibodies, receptors, library, immune cells, nucleic acids.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,686,292 A (SCHWALL et al) 11 November 1997, col. 4, lines 20-24; col. 5, lines 44-53; col. 6, lines 15-50; col. 20, lines 1-5	38, 40
X	WO 94/28391 A1 (IMMUNEX CORPORATION) 08 December 1994, page 32, lines 5-12; page 33, Example 4.	13-15, 38-40
Y		1-12, 16-37, 41
Y, P	US 5,753,516 A (HEAGY et al) 19 May 1998, col. 6, lines 41-53; col. 7, lines 11-62; col. 12, line 1 up to col. 13, line 65.	1-41
A	WO 93/14188 A1 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 22 July 1993, entire document.	1-41

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 MARCH 1999	Date of mailing of the international search report 13 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer T. WESSENDORF Telephone No. (703) 308-0196 JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/01331

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94/25571 A1 (BAXTER INTERNATIONAL, INC.) 10 November 1994, entire document.	1-41
A	WO 93/18136 A1 (CYTOMED, INC.) 16 September 1993, entire document.	1-41